

THE INFLUENCE OF CARRAGEENANS ON THE CELLS AND PATHWAYS OF
HAEMOSTASIS AND THEIR ROLE IN IMMUNOSUPPRESSION

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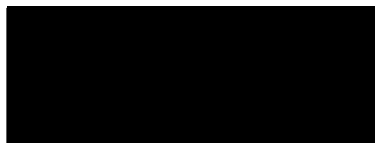
This thesis is presented to the Council for National
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Dundee College of Technology Q2/D.2
and Aberdeen University

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To my family

I hereby declare that this Thesis has been composed by myself and has not been presented in application for any previous Degree. The work of which this is a record has been done by myself and all other sources of information have been acknowledged.



Alison Morrison

ERRATA

p. 48 Additional sentence to paragraph 2 : Spectra for the remaining carrageenans are not included.

p. 48a Title should read: Infra-red spectrum of alkali-modified λ -carrageenan.

p. 50 Column 4 headed Sulphate should read Ester Sulphate.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS	PAGE NO.
ABBREVIATIONS	
I ABSTRACT	
II INTRODUCTION	
1. CARRAGEENANS	
1.1 Structure and properties	1
1.2 The molecular architecture of carrageenans	3
1.3 Chemically related mammalian polysaccharides	5
1.4 Physical properties of carrageenans	7
1.4.1 Chemical structure	7
1.5 Carrageenans as food additives	9
1.6 Pharmacological effects of carrageenans	9
1.6.1 Absorption of carrageenan from the gastro-intestinal tract	9
1.6.2 Toxicity of carrageenan	12
1.6.3 Effects of carrageenan on blood clotting	13
1.6.4 Inflammatory responses to carrageenan	13
2. HAEMOSTATIC PATHWAYS	
2.1 Introduction	15
2.2 The intrinsic pathway of coagulation	15
2.2.1 Thrombin-catalysed cleavage of fibrogen to fibrin	22
2.3 Regulation of haemostasis	23
2.4 The kallikrein-kinin system	26
2.5 Plasma fibrinolytic system	27
2.6 Platelets in blood coagulation	30
2.7 Plasma lipoproteins	32
2.8 Interrelationship of blood coagulation, kinin, complement and fibrinolytic pathway	33

3.	THE IMMUNE SYSTEM	
3.1	The immune response	36
3.2	B cells	38
3.3	T cells	40
3.4	Macrophages and antigen presentation	42
3.5	Immunosuppressive influence of carrageenan	44
4.	THE RODENT SYSTEM	
4.1	The rat	46
	Objectives of the study	47
III	MATERIALS AND METHODS	
	Polysaccharides	48
	Carrageenans	48
	Glycosaminoglycan	48
	Preparation of buffers and general reagents	51
	Route of polysaccharide administration	55
	Treatment of experimental data	56
	Preparation of plasma	56
	Platelet-rich plasma	56
	Platelet-poor plasma	57
	Modified plasma	57
	Factor XII-depleted plasma	57
	Antithrombin III-depleted plasma	58
	Preparation of serum	58
1.	COAGULATION STUDIES	
1.1	Clotting assays	60
1.1.1	Whole blood clotting assay	60
1.1.2	Partial thromboplastin clotting time assay	61
1.1.3	Russell's viper venom clotting assay	62
1.1.4	Thrombin clotting time assay	63
1.2	Amidolytic assays	63
1.2.1	Determination of Factor X in plasma	63
1.2.2	Determination of thrombin activity in plasma	64

2.	FIBRINOLYSIS	
2.1	Euglobulin precipitation of plasma	65
2.2	Inhibition studies	65
2.3	Preparation of fibrin plates	66
3.	KININ SYSTEM	
3.1	Determination of prekallikrein in plasma	67
4.	PLATELET STUDIES	
4.1	Platelet studies using platelet-rich plasma	68
4.2	Platelet aggregation	68
4.3	Platelet adhesiveness	69
5.	IMMUNOGLOBULIN STUDIES	
5.1	Enzyme-linked immunosorbent assay for rat immunoglobulin G	70
5.2	Haemagglutination assay	72
6.	LIPOPROTEIN STUDIES	
6.1	Determination of serum cholesterol	74
6.2	Lipoprotein separation	74
6.3	Determination of serum fraction cholesterol	76

IV RESULTS

A. In vitro studies

A.1	Blood clotting assays	77
A.1.1	Whole blood clotting time	77
A.1.2	Partial thromboplastin time clotting assay	77
A.1.3	Partial thromboplastin time in Factor XII-depleted plasma	77
A.1.4	Russell's viper venom clotting time	81
A.1.5	Russell's viper venom clotting time in Factor XII-depleted plasma	81
A.1.6	Russell's viper venom clotting time in antithrombin III-depleted plasma	81
A.1.7	Thrombin clotting time assay	84
A.1.8	Thrombin clotting time in antithrombin III-depleted plasma	84

A.1.9	Factor X (Factor Xa)-catalysed amidolytic assay	84
A.1.10	Thrombin-catalysed amidolytic assay	89
A.2	Fibrinolysis	
A.2.1	Euglobulin-derived fibrinolytic activity	94
A.2.2	Inhibitor studies with ϵ -aminocaproic acid	94
A.2.3	Inhibitor studies with flufenamic acid	94
A.3	The kallikrein-kinin system	
A.3.1	Amidolytic assay for carrageenan derived kallikrein activation	98
A.4	Blood platelet studies	
A.4.1	Effect of carrageenan on platelet aggregation	100
A.4.2	Effect of carrageenan on adenosine 5'-diphosphate-induced aggregation	100
A.4.3	Effect of carrageenan on thrombin-induced aggregation	100
B.	<u>Ex vivo</u> studies	
Route and dose of carrageenan administered		107
B.1	Blood clotting assays	
B.1.1	Partial thromboplastin clotting time assay	108
B.1.2	Russell's viper venom clotting time	108
B.1.3	Thrombin clotting time assay	108
B.1.4	Amidolytic activity of Factor X (Factor Xa)	112
B.2	Fibrinolysis	
B.2.1	Euglobulin derived fibrinolytic activity	114
B.2.2	Inhibitor studies with ϵ -aminocaproic acid	114
B.2.3	Inhibitor studies with flufenamic acid	117
B.3	Kallikrein-kinin system	
B.3.1	Amidolytic assay for carrageenan derived prekallikrein activation	119

B.4 Platelet studies	
B.4.1 Platelet counts in platelet-rich plasma	121
B.4.2 Adenosine 5'-diphosphate-induced aggregation	121
B.4.3 Thrombin-induced aggregation	125
B.4.4 Platelet adhesiveness studies	129
B.5 The immune response	
B.5.1. Antigen concentration in serum	135
B.5.2 Antibody response to 1-carrageenan admin- istration	135
B.5.3 Total immunoglobulin levels	138
B.5.4 The time of carrageenan injection in relation to antigen	138
B.6 Lipoprotein assay	
B.6.1 Serum lipoprotein studies	142
V DISCUSSION	
1. Blood clotting	148
2. Fibrinolysis	155
3. Kallikrein-kinin system	159
4. Platelet aggregation studies	162
5. The immune system	168
6. Lipoprotein studies	173
VI CONCLUSIONS	178
VII BIBLIOGRAPHY	181

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ABBREVIATIONS

a,f	Clotting factor subscript a represents activated factors; subscript f represents fragmented clotting factors following activation
ADP	Adenosine 5'-diphosphate
AT III	Antithrombin III
C $\bar{1}$ inhibitor	An inhibitor of the first component of complement
cyclic AMP	cyclic adenosine 3',5'-monophosphate
ϵ -ACA	ϵ -aminocaproic acid (6-amino hexanoic acid)
GAG	Glycosaminoglycan
HDL	High density lipoprotein
HMWK	High molecular weight kininogen
IDL	Intermediate density lipoprotein
IgG	Immunoglobulin G
LDL	Low density lipoprotein
MEM	Minimal essential media
PBS	Phosphate-buffered saline
pNA	paranitroaniline
PPP	Platelet-poor plasma
PRP	Platelet-rich plasma
PTT	Partial thromboplastin time
RVV	Russell's viper venom
SRBC	Sheep red blood cells
VBS	Veronal-buffered saline
VLDL	Very low density lipoproteins

THE INFLUENCE OF CARRAGEENAN ON THE CELLS AND PATHWAYS OF HAEMOSTASIS AND THEIR ROLE IN IMMUNOSUPPRESSION

Alison I. Morrison

I ABSTRACT

Carrageenans are a group of galactan sulphates derived from marine Rhodophyceae. These polymers have been shown to exert a variety of pharmacological effects, several of which involve alterations in both immune and haemostatic responses. Carrageenans were studied for the following reasons:

1. To observe the effects of defined carrageenan fractions on blood coagulation using plasma obtained from polysaccharide treated and untreated rats. Both in vitro and ex vivo samples were then subjected to amidolytic and functional clotting assays. Results obtained suggest that carrageenan is able to inhibit the activity of Factor XII, Factor I and thrombin. Unlike the known activities of the anticoagulant heparin, carrageenan activity was noted in the absence and presence of antithrombin III. The efficacy of carrageenan fractions to exert their particular anticoagulant response may reflect the ability of these polymers through their polyelectrolyte nature to form macromolecular complexes with respect to plasma proteins, thus neutralising their effectiveness in the clotting cascade.
2. Activation of the coagulation pathway also results in the activation of the fibrinolytic response. This activation can take place as a result of Factor II-dependent activation. Results obtained for the fibrinolytic response indicate that carrageenan did inhibit the fibrinolytic pathway when tested in vitro. However, plasma samples obtained from carrageenan treated animals were found to have enhanced fibrinolytic activity.
3. The kallikrein-kinin pathway, like fibrinolysis, may be triggered through Factor XII activation. In vitro results suggest that carrageenan inhibits prekallikrein activation while ex vivo an initial potentiation was observed.
4. In the formation of a haemostatic plug, blood platelets play an essential role. The carrageenan per se in vitro had no direct effect on blood platelets. In the presence of aggregating agents adenosine 5'-diphosphate and thrombin, carrageenan inhibits the platelet aggregation response. Platelet-rich plasma obtained from carrageenan treated rats suggests that the galactan sulphate may induce thrombocytopenia. Platelets from carrageenan treated rats were observed to have a reduced response to adenosine 5'-diphosphate and thrombin-induced platelet aggregation.
5. Carrageenan-lipoprotein effects were investigated to correlate lipoprotein levels with blood coagulation. Results suggest that macroion complex formation between lipoprotein lipase receptors and carrageenan may result in a release of the lipase into the blood stream.
6. Finally, the immunosuppressive effect of carrageenan on the rat model was investigated. Preliminary studies suggest that the polyelectrolyte nature of the carrageenan may influence its role as an immunosuppressive agent.

II INTRODUCTION

1. CARRAGEENANS

1.1 Structure and properties

Carrageenan is the name given to a galactan sulphate of high molecular weight extracted from marine algae such as Chondrus crispus. This alga is also known as carrageenin, "Irish moss" or "carragheen moss" from Carragheen, Waterford in Ireland. The name carragheen, to designate the extract from Chondrus crispus, was first used by Stanford in 1862. The term carrageenan is more recent and was adopted by several authors after 1950 (American Chemical Society).

Carrageenans are isolated by aqueous extraction from various members of marine Rhodophyceae. The principal species commercially harvested are illustrated in Fig. 1.1.1. The polysaccharide structure comprises alternating α -(1 \rightarrow 3), β -(1 \rightarrow 4) linked D-galactopyranose units. Carrageenans are highly sulphated with an ester sulphate content estimated 20-40% by dry weight.

In 1953 Smith and Cook showed that carrageenan extracted from C. crispus could be fractionated to yield lambda (λ -) and kappa (κ -) carrageenan as characterised by different solubilities in aqueous solution.

κ -Carrageenan was that fraction precipitated from aqueous solution by the addition of potassium chloride up to a concentration of 0.25M. The fraction that remained in solution under the above conditions was designated λ -carrageenan.

Figure 1.1.1

CLASSIFICATION OF RHODOPHYCEAE

CLASS	Rhodophyceae			
ORDER	Gigartinales			
FAMILY	Gigartinaceae		Solieriaceae	Hypneaceae
GENUS	Gigartina	Chondrus	Eucheuma	Hypnea
SPECIES	<u>G. stellata</u>	<u>C. crispus</u>	<u>E. spinosum</u>	<u>H. musciformis</u>
	<u>G. pistillata</u>	<u>C. ocellatus</u>	<u>E. cottonii</u>	
	<u>G. acicularis</u>			
	<u>G. radula</u>			

κ -Carrageenan is composed of sulphated D-galactose and 3,6-anhydro-D-galactose residues in approximately equimolar concentrations having a branched structure with molecular weight between $1.8 - 3.2 \times 10^5$ daltons (Smith, Cook and Neal, 1954; Smith, O'Neil and Perlin, 1958). The λ -carrageenan fraction was found to consist almost entirely of sulphated D-galactose with a molecular weight range between $4 - 7 \times 10^5$ daltons (Smith, O'Neil and Perlin, 1958).

One other structure, iota (1-) carrageenan was found in C. crispus in small quantities. 1-Carrageenan may be a precursor of κ -carrageenan by the enzymic formation of an anhydrogalactose ring due to sulphate elimination (Peat, 1981).

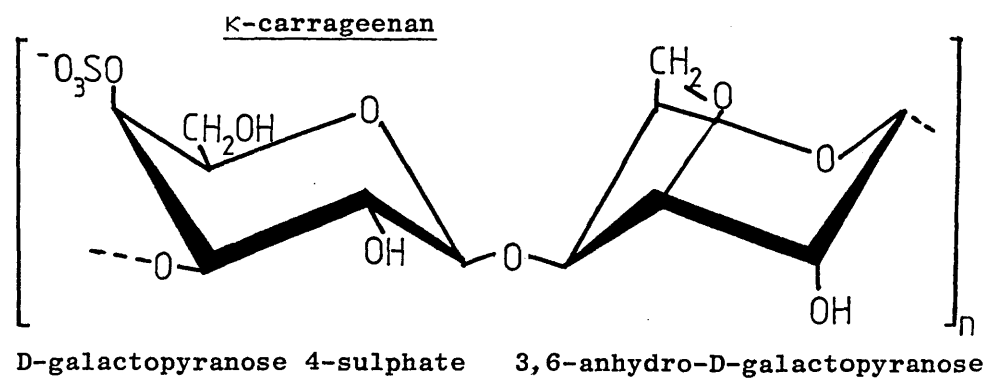
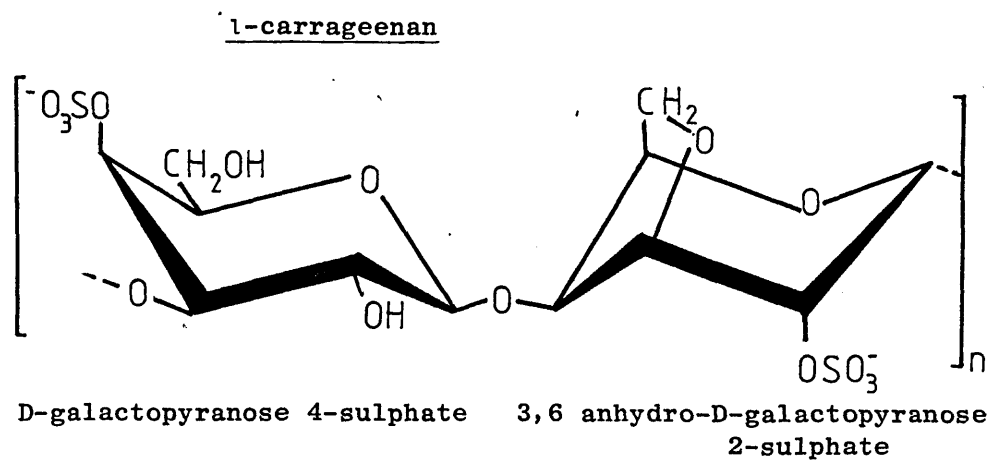
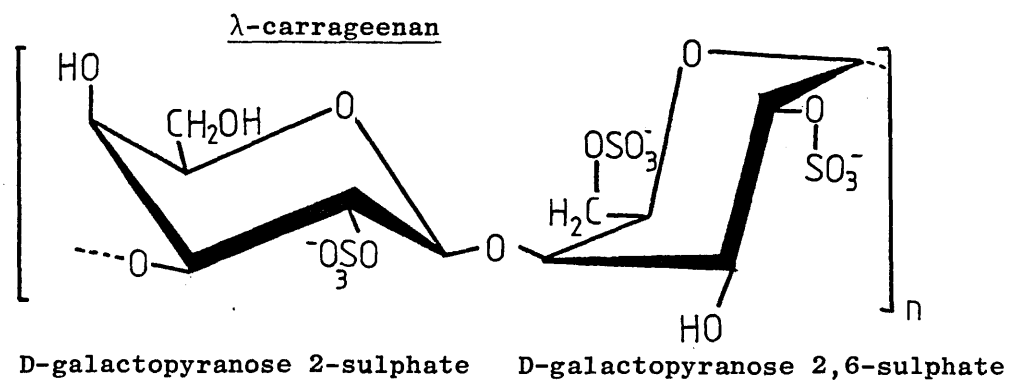
The principal source of commercial carrageenan is C. crispus. This species yields both κ - and λ -carrageenan fractions and forms the basis of a multi-million pound industry.

1.2 The molecular architecture of carrageenans

Commercially isolated fractions of carrageenans have been extensively characterised in the laboratory and their chemical composition reviewed by Yaphe (1959), Rees (1961) and Percival (1972). From these studies it has been found that κ -carrageenan may be represented by the idealised structure illustrated, Fig. 1.2.1. It is composed of α -(1 \rightarrow 3)-linked D-galactopyrano-4-sulphate and β -(1 \rightarrow 4)-linked 3,6-anhydro-D-galactopyranose units (Anderson, Dolan and Rees, 1968), with the α -(1 \rightarrow 3) glycosidic and β -(1 \rightarrow 4) glycosidic bonds alternating. Similarly, λ -carrageenan exhibits heterogeneity and Rees (1969) suggested that the name λ -carrageenan be reserved for material with the idealised structure composed of α -(1 \rightarrow 3)-linked D-galactopyranose-2-sulphate and β -(1 \rightarrow 4)-linked D-galactopyranose-2,6-disulphate, Fig. 1.2.1.

Figure 1.2.1

Idealised structures of lambda, iota and kappa carrageenan



The idealised structure of 1-carrageenan may be represented as shown in Fig. 1.2.1. The polymer has a disaccharide repeat unit of α -(1 \rightarrow 3)-linked D-galactopyranose-4-sulphate and β -(1 \rightarrow 4)-linked 3,6-anhydro-D-galactopyranose-2-sulphate (Anderson, Dolan and Rees, 1968). Physical-chemical analysis of macromolecular extracts revealed the existence of chemical heterogeneity within individual species.

1.3 Chemically related mammalian polysaccharides

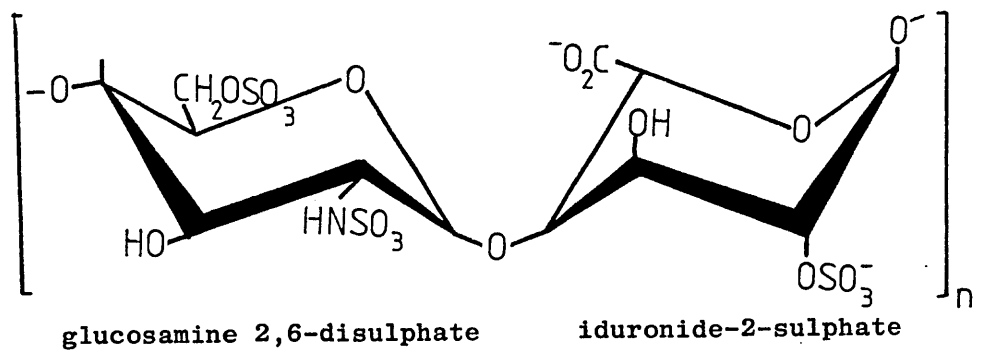
The galactan sulphates isolated from the seaweed are not unique and similar polymers have been extracted from a variety of organisms. These polymers, named glycosaminoglycans (GAGS), are primarily found in mammals and have a wide range of functions from providing physical support to a possible participation in blood clotting.

One such GAG is heparin, a long unbranched polysaccharide comprised of repeat units of glucosamine 2,6-disulphate and iduronide-2-sulphate (Casu et al., 1979), Figure 1.3.1. Sulphate and/or carboxyl groups present on the disaccharide unit make the chains strong polyanions at pH 7.4 and this results in some of its anticoagulant activity (Rees, 1975). Unlike other GAGS, heparin is not a structural component, but occurs as an intracellular component in skin, lung, liver, gastric mucosa and umbilical cord. With respect to its commercial importance, heparin is isolated from bovine liver capsule and pig gastric mucosa.

Mammalian GAGS and polysaccharides from red algae exhibit many similarities in chemical and physical properties and an obvious inference is that, in at least some of their pharmacological effects, carrageenans mimic the actions of their mammalian counterparts.

Figure 1.3.1

Idealised structure of heparin



1.4 Physical properties of carrageenans

1.4.1 Chemical structure

Carrageenans, with the exception of λ -carrageenan, form helices in solution (McKinnon, Rees and Williamson, 1969). In the case of λ -carrageenan, the chains are linked by inter-chain hydrogen bonding with the sulphate esters positioned on the outside of the helix (Williamson, 1970; Morris et al., 1978). The flexibility of this polymer is restricted to rotations around the four glycosidic bond angles and the 3,6-anhydro-D-glycosidic bond angles. The 3,6-anhydro-D-galactopyranose residues increase flexibility of the polymer when in solution. κ -Carrageenan forms a triple helix structure and in the formation of a matrix, straight chain λ -like regions occur. κ -Carrageenan forms the most viscous solution of the three types investigated. λ -Carrageenan, however, forms a rigid straight chain and forms a less viscous solution.

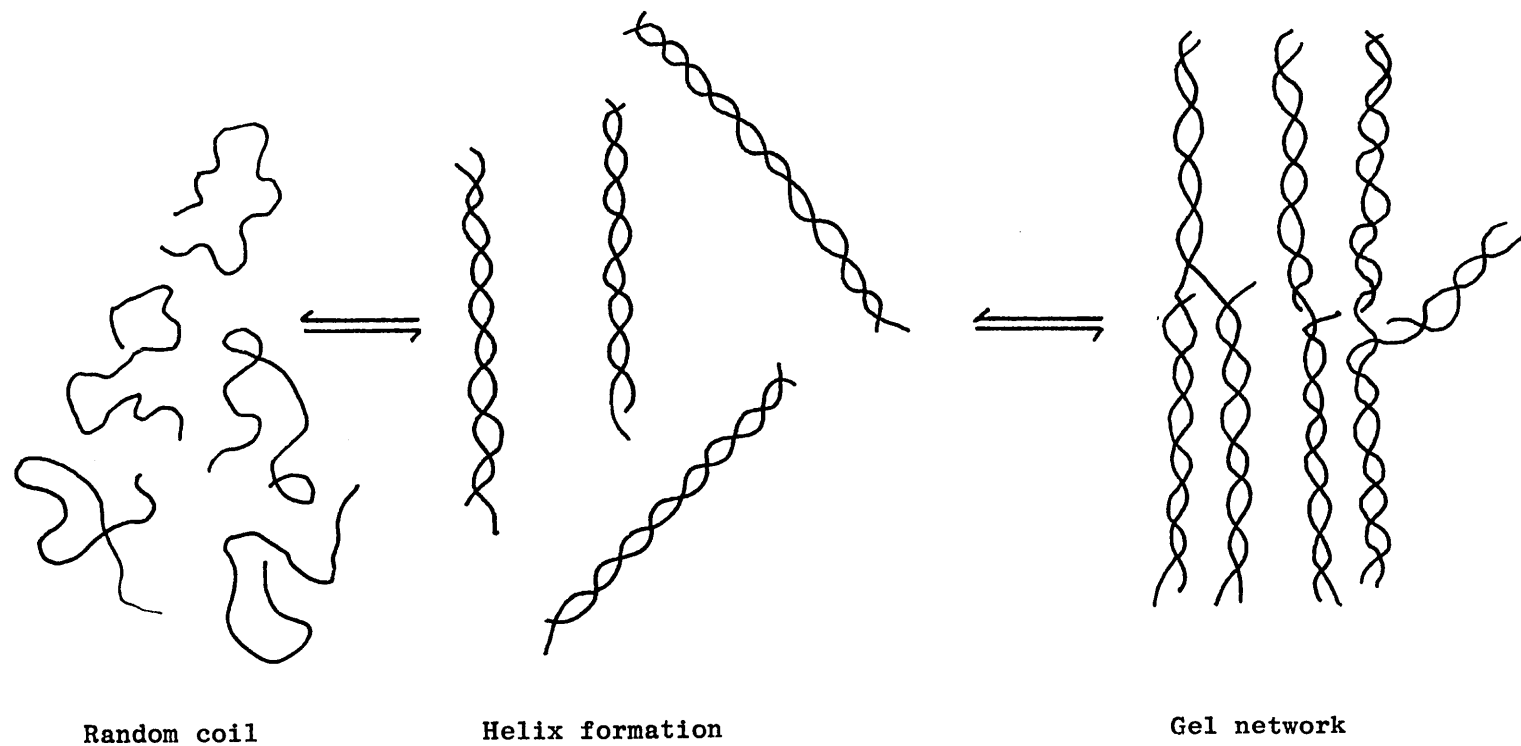
Criteria such as the freedom of rotation, chain-chain interaction and ionic radius of the cations involved in the overall structure of the gel matrix may influence the physiological and chemical properties of these polymers (Kindness, 1979).

1.4.2 Gel formation

As previously mentioned some carrageenans form helices under certain conditions of temperature and concentration. Such interactions can act as tie points to give a three dimensional gel network (Fig. 1.4.2.1). Should such a gel network occur in vivo then water and ion retention may occur. It may also mediate in the passage of molecules in and out of the cells in the manner of an ion exchange molecular sieve (Rees, 1969, 1972).

Figure 1.4.2.1

Idealised gel network formation



One important type of gel interaction is that with proteins. This is of considerable relevance to the food industry and may offer an insight into the physiological effects of carrageenans. The properties of glycosaminoglycans and their influence on protein, ion and water binding have been reviewed by Lindahl and Hook (1978).

1.5 Carrageenans as food additives

The use of carrageenans as food additives dates back many years to when seaweed was pulverised with milk to give a jelly-like food (Sauvageau, 1920). However, since 1937 carrageenan has been produced and sold as a food emulsifier and stabiliser on a commercial scale (Institute of Food Technologists Panel, 1973).

Due to the results of research carried out on the pharmacological effects of high concentrations of carrageenan, the World Health Organisation's Expert Committee on Food Additives recommended in 1969 that daily intake of carrageenan should not exceed 50 mg kg^{-1} , per day. The concentration of carrageenan required to stabilise foods ranges from 0.005 to 1.2% on a dry weight basis and thus the intake per individual is far below the recommended level.

Carrageenans have been used as emulsifiers, stabilisers and thickeners because of their ability to stabilise proteins, especially milk proteins (Hansen, 1966; Hansen and Lin, 1968; Lin, 1977), in foods such as indicated in Fig. 1.5.1.

1.6 Pharmacological effects of carrageenans

1.6.1 Absorption of carrageenans from the gastro-intestinal tract

Since the molecular weight of carrageenans used in food preparation is high, it was hoped that the polymer would not be absorbed through the

Figure 1.5.1

CARRAGEENAN APPLICATIONS

Gelling Agent	Milk	jellified milk acidified milk puddings aerated desserts
	Water	low calorie jams jellied fruits water gels meat aspics pet food air freshners
Thickening Agent	Milk	dessert creams custard yoghurt
	Water	cream soup tomato ketchup cosmetics toothpaste
Stabilisers	Milk	chocolate ice creams whipped creams
	Water	mayonnaise salad dressing squash

intestinal wall. To date, the exact nature of carrageenan absorption is unclear. The addition of carrageenan to the diet of monkey (Beattie et al., 1976; Abraham, Goldberg and Coulson, 1972; Grasso et al., 1973), pig (Poulsen, 1973), rat (Nilson and Wagner, 1959; Hawkins and Yaphe, 1965; Grasso et al., 1973), mouse (Grasso et al., 1973) and hamster (Grasso et al., 1973) resulted in carrageenan being found in faeces.

Work carried out by Nilson and Wagner (1959) using rats indicated that a carrageenan level of above 10% of dry diet retarded growth and resulted in liver abnormalities. Anderson and Soman (1966) showed that high molecular weight carrageenan was not absorbed through the gut of the guinea pig and could not be detected in the blood or urine. Further studies by Watt and Marcus (1969), in the same species, suggested that high molecular weight carrageenan caused ulceration of the caecum and colon. Similar results were observed with rabbits (Grasso et al., 1973).

Results would appear to be greatly differing; however, the outcome of these experiments may be related to the types of food stuffs normally consumed by the experimental animal and thus may reflect the influence of the endogeneous gut flora. Large macromolecules are known to be absorbed through neonatal and foetal small intestine (Freeman and Kim, 1978). Long-term feeding of carrageenan was found to affect rat foetal development (Collins, Blank and Prew, 1977).

In the human intestine, bacteria with the ability to break down carrageenan may exist. Bacteria having a carrageenolytic effect have been found in the intestine and this may be a direct effect of the usage of carrageenan in food (Yaphe and Baxter, 1956; Weigl and

Yaphe, 1966a,b).

The pharmacological effects of degraded carrageenan are controversial. Carrageenan fragments with mean molecular weights ranging from 4×10^3 - 2×10^4 daltons were commercially prepared for the treatment of peptic and duodenal ulcers in humans. It was demonstrated that carrageenan had no adverse effect on the human colon over an extended period of 2 years (Bonfils, 1970). Ingested carrageenan was found to have a lowering effect on blood cholesterol levels in chickens and rats (Fahrenbach, Riccardi and Grant, 1966; Ershoff and Wills, 1962). In this instance, the carrageenan may have caused a shift from low density lipoprotein (the density of lipoprotein involved in atherosclerosis), to high density lipoproteins which are removed from the body by the liver.

Thus carrageenan has been found to have many, if conflicting, effects on physiological body functions of animals following ingestion. Results would suggest that the usage of carrageenan in food stuffs should be limited until the nature of long-term influences may be determined.

1.6.2 Toxicity of carrageenan

Wilson et al. (1978) showed that an injection of 200 mg kg^{-1} of carrageenan into the rat peritoneal cavity resulted in toxicity. The most toxic formation found was K-carrageenan which caused disseminated intravascular coagulation. Injection of the other carrageenan fractions, namely λ - and ι -carrageenan, also resulted in a toxic effect but to a lesser degree. Fowler, Simpson and Thomson (1980) similarly showed that the administration of carrageenan to rats produced tissue damage with focal blanching of the liver and splenomegally evident post mortem.

1.6.3 Effects of carrageenan on blood clotting

In 1954 Ratnoff observed that plasma incubated with carrageenan and activated with thrombin took a prolonged time to clot depending on the concentration of carrageenan. Later, work by Hawkins and Leonard (1963) indicated that carrageenan and carrageenan fractions λ - and κ - prolonged plasma clotting time, λ - being the more efficient inhibitor of clotting. Debate over the exact nature of carrageenan anticoagulant activity resulted. Experiments carried out by other workers showed that the anticoagulant activity of carrageenan may be mediated via early stages of the coagulation pathway (Anderson and Duncan, 1965). The fact that carrageenan activity was concentration- and structure-dependent was verified with high concentrations of carrageenan prolonging blood clotting time whilst low concentrations were procoagulant.

These studies were mainly carried out on human plasma samples and to date there is no clear evidence of exactly at what point of the coagulation cascade system carrageenan exerts its effects. Experimental work carried out by Kindness, Long and Williamson (1980a), illustrated that on human plasma test systems carrageenans were anticoagulant.

1.6.4 Inflammatory responses to carrageenan

The inflammatory response to carrageenan was first observed by Winter, Risley and Nuss (1962). Carrageenans are now used extensively as producers of oedema against which potential anti-inflammatory drugs are screened and evaluated (Rocha e Silva, 1970; Schorlemmer, 1977; Damus, 1980a; Labreque, Doré and Bélanger, 1981). In experimental animals the hind paw receives a carrageenan dose subcutaneously, and the paw is then observed for swelling. Inflammation induced by carrageenan

was found to be suppressed by soybean trypsin inhibitor as well as by the administration of Aprotinin (van Arman et al., 1965; Di Rosa and Sorrentino, 1968). Both these compounds inhibit activation of the kallikrein-kinin system, thus preventing the production of vasoactive peptides termed kinins which are known to mediate inflammation and pain production.

Di Rosa and Sorrentino (1970) showed that animals injected with carrageenan had decreased arterial blood pressure thus confirming kinin production. Carrageenan-induced hypotension was also found by Damus (1979) but this effect was not present in thrombocytopenic rats; thus it may be speculated that platelets are causally involved in the hypotensive response.

A considerable amount of work has been carried out in vitro to investigate the influence of carrageenans on blood platelets (McMillan, McIntyre and Gordon, 1979; Vargaftig et al., 1980; Deflandre and Damus, 1980; Kindness, Williamson and Long, 1980b). Carrageenans have been shown to aggregate human and rabbit platelets in vitro (Gordon, McIntyre and McMillan, 1977; Vargaftig, 1977; Kindness, Williamson and Long, 1980b). Thus the aggregation and release of platelet contents may contribute to inflammatory mechanisms.

2. HAEMOSTATIC PATHWAYS

2.1 Introduction

Blood coagulation, whether in vivo or in vitro, occurs as a result of a cascade system involving the interaction of nearly a dozen proteins, and giving rise to the formation of network of fibrin.

Over the many years of research into the processes of blood clotting the proteins have been called by a variety of names. However, the International Committee on Haemostasis and Thrombosis allocated a Roman numeral to each of the components of the coagulation pathway (Figure 2.1.1).

The formation of a blood clot can be triggered by two pathways sharing a common junction at Factor X, and resulting in the formation of an insoluble fibrin clot, (Fig. 2.1.2). As can be seen from Fig. 2.1.3, the intrinsic pathway arises from the activation of Factor XII without tissue damage having taken place. In contrast, activation of the extrinsic pathway is the direct result of damage to a blood vessel whereby tissue thromboplastin is released. Tissue thromboplastin in conjunction with exposed collagen and Factor VII activates Factor X. The exact role of tissue thromboplastin is not known but the enzymically active groups responsible for Factor X activation appear to be located on Factor VII. Both pathways require the additional components of platelet phospholipid and plasma calcium in order to form the insoluble fibrin clot.

2.2 The intrinsic pathway of coagulation

The initiation of the intrinsic pathway involves the participation of a number of proteins and contact with a suitable surface. Identification

Figure 2.1.1

CLOTTING FACTORS

Factor Number	Synonym used
I	Fibrinogen
II	Prothrombin
III	Tissue thromboplastin
IV	Calcium ions
V	Proaccelerin
VII	Factor VII
VIII	Antihæmophilic factor
IX	Christmas factor
X	Stuart factor
XI	Plasma thromboplastin antecedent
XII	Hageman factor
XIII	Fibrin-stabilising factor
Prekallikrein	Fletcher factor
H.M.W. Kininogen	High molecular weight Kininogen, Fitzgerald factor

Figure 2.1.2

The extrinsic pathway of coagulation

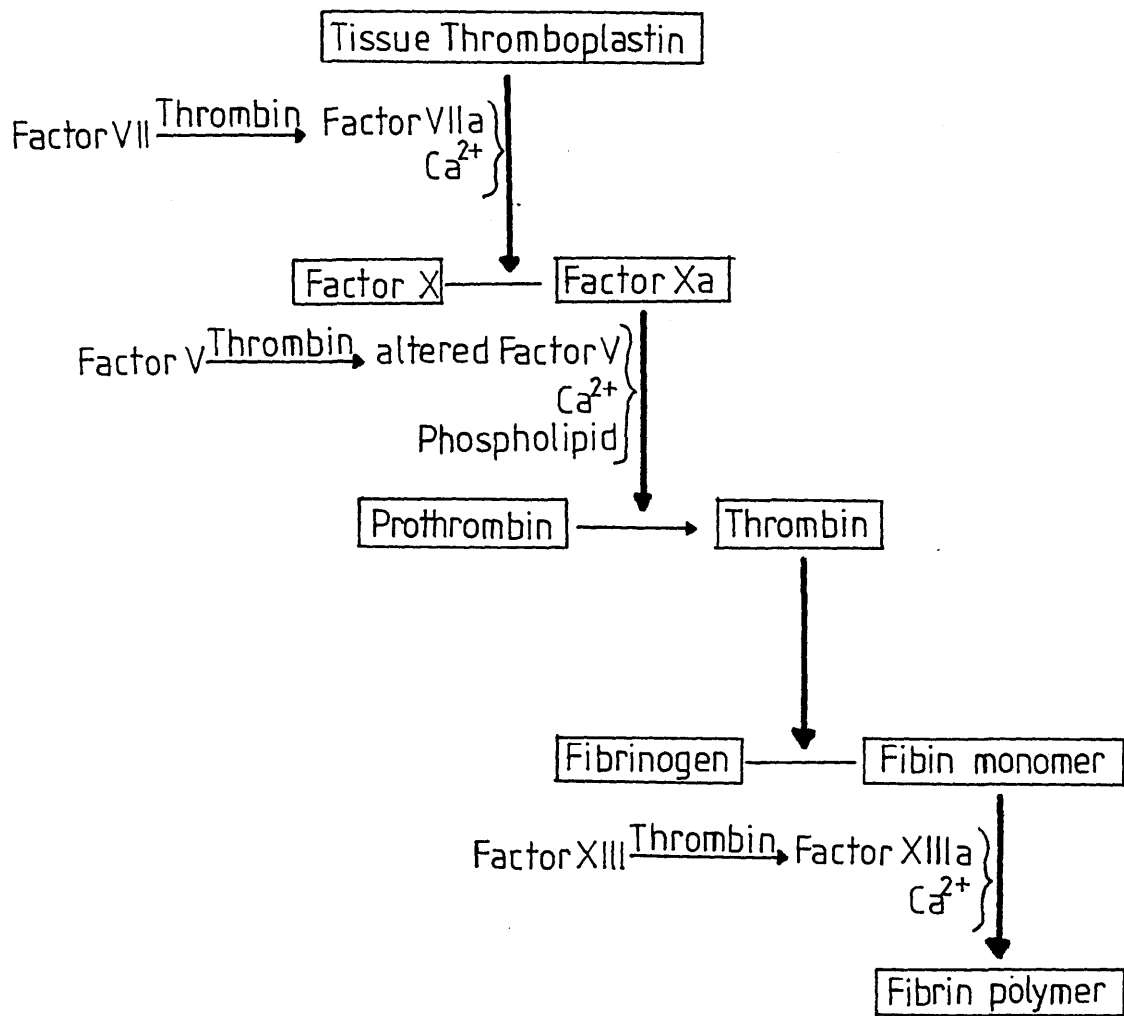
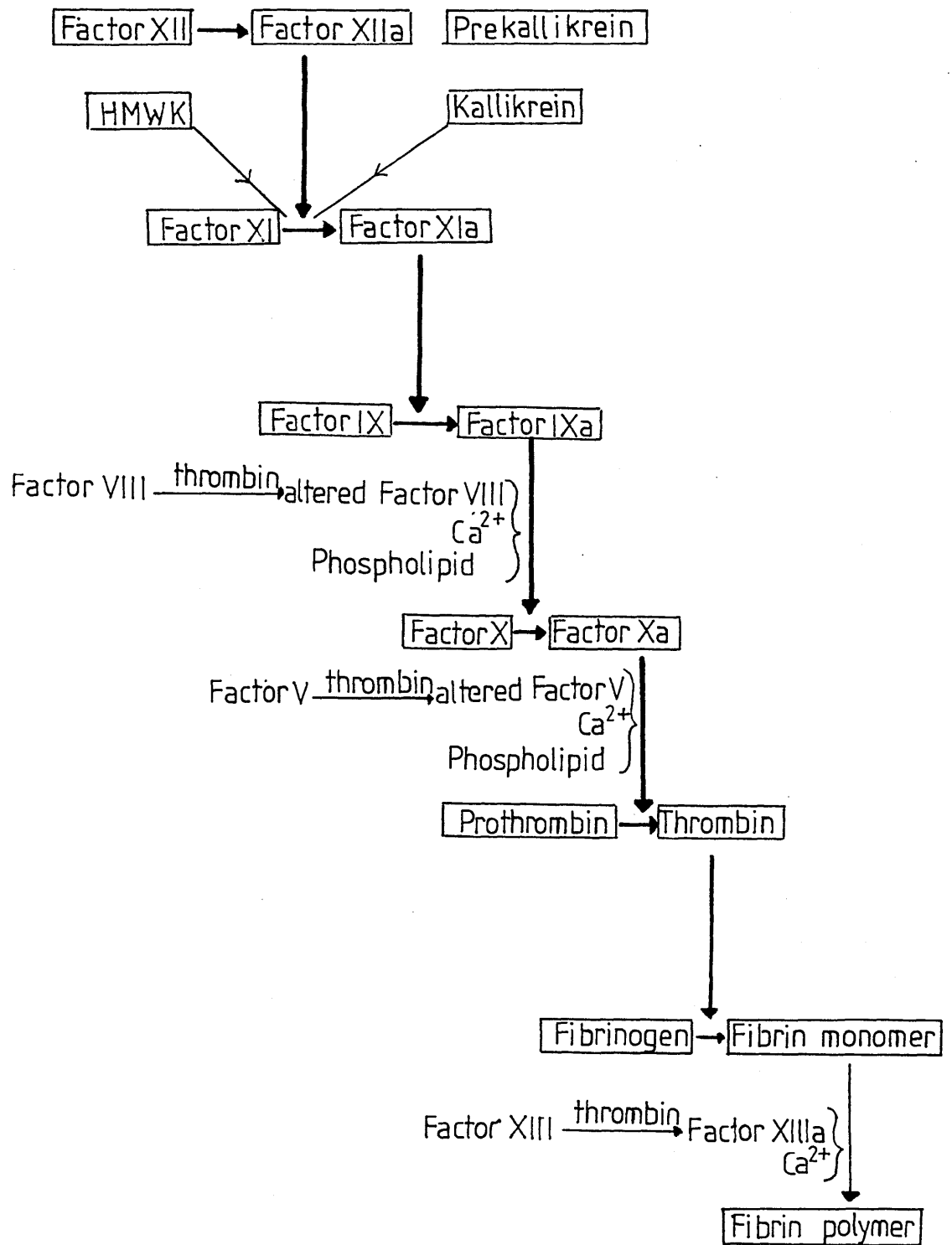


Figure 2.1.3

The intrinsic pathway of coagulation



of the proteins participating in the coagulation cascade arose from studies of individuals with familial bleeding tendencies.

The initial factor required for intrinsic coagulation is Factor XII, first isolated by Ratnoff and Colopy (1955). Hageman deficiency, a deficiency of Factor XII, was observed in individuals having a prolonged bleeding time. The site of synthesis of Factor XII is unknown; however patients with hepatic cirrhosis have decreased plasma concentrations (Saito et al., 1976).

Experimentally, activation of Factor XII occurs on contact with a large variety of negatively-charged surfaces such as glass or kaolin (Ratnoff and Rosenblum, 1958), collagen (Wilner, Nossel and LeRoy, 1968), platelet membranes (Walsh, 1972) and endothelial cells (Wiggins et al., 1980). It has been proposed that the binding of Factor XII to a negatively-charged surface results in a conformational change in the molecule exposing the active site required for enzymic activity. This hypothesis was confirmed by circular dichroism spectroscopy (McMillin et al., 1974; Fair et al., 1977).

Apart from solid phase activation of Factor XII on contact with negatively-charged surfaces, fluid phase activation occurs when Factor XII is in contact with proteinases such as kallikrein or plasmin, (Ogston, 1981; Donaldson and Harrison, 1982). This activation results in the proteolytic cleavage of Factor XII and the fragments produced have the property of converting prekallikrein to kallikrein (Kaplan and Austen, 1971; Cochrane and Wuepper, 1971). The clot-promoting activity of this active fragment is much less than that of the parent molecule (Revak et al., 1974). Debate exists as to whether cleavage of Factor XII is required for clot-promoting properties. In 1979, Ratnoff

and Saito carried out experiments that indicated that there was no cleavage within the active Factor XII molecule. The alternative view, however, is that Factor XII activation requires cleavage within a disulphide loop to form a two chain molecule (Griffin, 1977; Meier et al., 1977).

Studies carried out on surface-bound Factor XIIa and the free fraction have shown differences in their ability to catalyse the cleavage of radiolabelled prekallikrein and Factor XI. Both were capable of catalysing the cleavage of and thereby activating prekallikrein but only the surface-bound molecule caused the cleavage of Factor XI. Hence these results would indicate that initiation of the intrinsic pathway is a localised process occurring at the site of contact activation (Revak et al., 1978).

Factor XIIa activates Factor XI; however, experiments by Schiffman and Lee (1974) showed that purified Factor XIIa and Factor XI could not induce clot promoting activity. It is now recognised that both prekallikrein and HMWK are required for the activation of Factor XI but the exact nature of their function has not yet been clarified (Donaldson, personal communication). These requirements for clot-promoting activity were recognised in patients deficient in prekallikrein (Hathaway, Belhasen and Hathaway, 1965) or HMWK (Saito et al., 1975).

HMWK accelerates activation of Factor XI by Factor XIIa, activation of prekallikrein by Factor XII and activation of Factor XII by kallikrein (Griffin and Cochrane, 1976). Thus, it is thought that Factor XIIa and HMWK form a complex on a negatively-charged surface which induces a conformational change in the Factor XI, rendering it susceptible to proteolytic cleavage. The surface-bound Factor XIIa activates

prekallikrein and Factor XI. The generated kallikrein then activates more Factor XII thus increasing the quantity of Factor XIIa (Ogston, 1981). Meier et al. (1977) found that kallikrein-activated Factor XII by positive feedback: HMWK did not aid Factor XII binding to a surface but potentiated its ability to activate prekallikrein and Factor XI. Factor XIIa did not require prekallikrein but HMWK was required for the interaction of Factor XIIa and Factor XI (Ratnoff and Saito, 1979).

Fujikawa et al. (1979) raised the possibility of an alternative pathway of Factor XII activation in which Factor XII would be readily activated by kallikrein in the presence of dextran sulphate, even in the absence of a surface such as kaolin. Once Factor XI has been activated, it converts the inactive precursor Factor IX to its active form. This process involves a proteolytic cleavage to reveal the active site.

Factor IX forms a complex with phospholipid, obtained in the form of a platelet surface, calcium ions, Factor VIII and inactive Factor X. The Factor VIII may be present in the circulating plasma or bound to the platelet surface. A deficiency of Factor VIII causes excessive bleeding and it is thought that Factor VIII is required for platelet adhesion to subendothelial tissue.

Factor IXa and Factor VIII are adsorbed onto the phospholipid surface in the presence of calcium and in this form activate Factor X. The phospholipid may provide a surface on which Factor IXa and Factor VII align themselves in a suitable conformation to activate Factor X. The calcium ions form positively-charged bridges between the negatively-charged phospholipid and proteinases (Baugh and Hougie, 1979).

Thrombin, which is present in small concentrations in the plasma,

activates Factor VIII. If the concentration of thrombin increases, inactivation of Factor VIII occurs. This is an example of negative feedback where inhibition of the reaction prevents thrombotic tendencies (Ogston, 1981).

Factor Xa, acting in concert with phospholipid and calcium ions, activates Factor V which is converted to thrombin by proteolytic cleavage. Thrombin is the enzyme responsible for the conversion of fibrinogen to fibrin.

2.2.1 Thrombin-catalysed cleavage of fibrinogen to fibrin

Fibrinogen is a dimer composed of two symmetrical subunits linked by disulphide bridges, with two-fold symmetry (Blomback, Hessel and Hog, 1976). The half molecules each consist of three polypeptide chains ($A\alpha$, $B\beta$, γ), and the interchain disulphides occur as two clusters in the molecule, one in the N-terminal part and a similar cluster in the C-terminal area (Doolittle et al., 1979). Interchain disulphide loops occur in C-terminal portions of all three chains (Blomback and Blomback, 1972; Blomback, Hessel and Hog, 1976).

Factor Xa catalyses the cleavage of prothrombin to form active thrombin, a two chained molecule (Magnusson et al., 1976). Thrombin, like Factor X, Factor IV and Factor VII is dependent on vitamin K for its synthesis, and like Factor II, is synthesised in the liver. Vitamin K imparts the ability to adhere to phospholipid in the presence of calcium ions. Thus, a complex of Factor Xa, Factor VII and Factor II attaches itself to a phospholipid surface, probably platelet derived, via calcium ion bridges and the active enzyme thrombin is released from the phospholipid into the circulation (Magnusson et al., 1975).

Thrombin acts on fibrinogen to form initially a linear polymer and then a branched polymer with resultant cleaved fibrinopeptides. The last enzymic step in blood clotting centres around the strengthening of the fibrin-to-fibrin association by inter-molecular γ -glutamyl- ϵ -lysine bridges (Lorand, 1972). The fibrin-stabilising factor (Factor XIII) circulates as an inactive precursor which is converted to the active form in the presence of thrombin and calcium ions. The Factor XIII action results in the production of a cross-linked fibrin network (Lorand, 1972).

2.3 Regulation of haemostasis

The existence of control mechanisms within coagulation and platelet aggregation has been under investigation but a lot less is known about these systems than for contact activation.

Results from experiments on the enzymic action of thrombin on fibrinogen indicated that the blood possessed the ability to inactivate thrombin (Schmidt, 1892). The substance responsible was later found to be Antithrombin (Moravik, 1905). Research in this field progressed slowly and only in the last fifteen years have the clinical consequences of Antithrombin deficiency been elucidated (Egeberg, 1965).

Antithrombin (AT III) is one of the proteinase inhibitors of blood, inactivating all the clotting enzymes except Factor VIIa. This inhibitory effect was found to be greatly accelerated in the presence of heparin, by a factor of 1000 (Quick, 1938).

AT III is a α_2 -globulin and comparison of amino acid sequence reveals a relatively high degree of homology with α_1 -antitrypsin, suggesting the two inhibitors may have a common ancestry (Nordenman, Nyström and Björk, 1977; Peterson et al., 1979). The binding of heparin

to AT III is dependent on ionic strength and pH may occur at one (Danielson and Björk, 1978; Björk et al., 1981), or two binding sites on the AT III molecule (Piepkorn, Lagunoff and Schmer, 1978).

Fluorescence studies suggest a low molecular weight fraction of heparin binds to AT III in molar ratio 1:1, while high molecular weight fractions appear to have two binding sites (Nordenman and Björk, 1978; Rosenberg et al., 1979).

The precise mode of heparin action on AT III is unknown but three models have been proposed. Heparin may bind to AT III, inducing a conformational change to facilitate thrombin binding. Once the thrombin is bound, the heparin is released and binds another AT III molecule (Rosenberg and Damus, 1973; Li, Fenton and Feinman, 1976; Marciniak and Gora-Maslak, 1982). Alternatively, heparin may bind to thrombin thus inducing a reaction with AT III (Machovich, Blasko and Palos, 1975). Lastly, heparin and thrombin may bind simultaneously to AT III and induce a conformational change whereupon heparin is released (Holmer, Söderström and Andersson, 1979).

AT III is also known to interact with other glycosaminoglycans, such as heparan sulphate and dermatan sulphate, but in each case a higher concentration of polysaccharide was required (Teien, Abilgaard and Hook, 1976; Hatton, Berry and Regoeczi, 1978; Kindness, Long and Williamson, 1979). Heparan sulphate apparently functions in a heparin-like manner, whilst dermatan sulphate exerts its anticoagulant effect in a manner independent of AT III activity. In view of the presence of these compounds in the vascular wall, these findings suggest their importance as stationary anticoagulants at the endothelial surface (Hatton, Berry and Regoeczi, 1978).

Relatively little is known about inhibitors other than AT III and the glycosaminoglycans. The serine proteinase inhibitors in plasma tend to have broad specificity but vary in their affinity towards the various enzymes.

The antiproteinase α_2 -macroglobulin (α_2 -M) binds to enzymes without blocking their active sites, permitting the cleavage of small molecular substrates. Steinbuch, Blatrix and Josso (1966) showed that α_2 -M inhibited the clotting activity of thrombin. Since then it has been shown that α_2 -M accounts for 25 per cent and AT III 70 per cent of total thrombin inhibiting action of plasma (Shapiro and Anderson, 1977; Josso, 1978). Thrombin inhibition is much more rapid in the presence of AT III than with α_2 -M (Abildgaard, 1969).

The inactivation of thrombin by α_1 -antitrypsin occurs at a low rate provided the initial thrombin concentration is high but this effect is much weaker than that of AT III or α_2 -M. As individuals with a deficiency of α_1 -antitrypsin have normal thrombin inhibitory activity in plasma and do not show a special tendency towards thrombosis, these in vitro anticoagulant effects appear to have little or no physiological relevance in vivo.

An inhibitor of the activated first component of the complement system, $\text{C}\bar{1}$ inhibitor, also inhibits several haemostatic enzymes of plasma: Factor XIIa (Forbes, Pensky and Ratnoff, 1970); kallikrein (Ratnoff et al., 1969); Factor XIa (Forbes, Pensky and Ratnoff, 1970) and the fibrinolytic enzyme plasmin (Schreiber, Kaplan and Austen, 1973). Complexes between $\text{C}\bar{1}$ inhibitor and the above proteinase excluding plasmin, have also been detected by Donaldson and Harrison (1982).

The antiproteinasases detailed above are required to regulate the pathways associated with coagulation, inflammation and fibrinolysis. It has been shown that negatively-charged galactan sulphates are capable of interacting with these antiproteinasases and thereby may modify the physiological activities associated with the haemostatic response (Ratnoff, 1954; Kindness, Long and Williamson, 1981).

2.4 The kallikrein-kinin system

During contact activation of plasma, Factor XII binds to negatively-charged surfaces together with HMWK and prekallikrein. The HMWK circulates, complexed at least in part to prekallikrein or Factor XI (Mandle, Colman and Kaplan, 1976; Thompson, Mandle and Kaplan, 1977), and is instrumental in bringing both prekallikrein and Factor XI to the surface (Wiggins et al., 1977). The orientation of Factor XII with prekallikrein leads to limited proteolytic cleavage of Factor XII and activation of both molecules, each serving as a substrate for the other (Weiss, Gallin and Kaplan, 1974). Cochrane and Revak (1980) concluded that contact activation of plasma may be divided into three phases; firstly, the activation of Factor XII by prekallikrein on the surface with HMWK acting as a cofactor orientating the molecules into the correct position for cleavage; secondly, this activation was followed by a rapid release of kallikrein into the fluid phase; finally, the continued conversion of prekallikrein to kallikrein by each surface-bound molecule of Factor XII.

Two forms of kininogen exist in plasma; these are low molecular weight kininogen (LMWK) and high molecular weight kininogen (HMWK) (Kaplan, 1979). HMWK is cleaved by kallikrein to produce the vasoactive peptide bradykinin and other fragments (Kaplan, 1979; Nakayaso and

Nagasaura, 1979). Bradykinin production may remove the cofactor for the conversion of prekallikrein to kallikrein and the conversion of Factor XII to Factor XIIa (Chan, Movat and Burrowes, 1979).

Plasma kallikrein has esterase activity and is inhibited by at least four different proteinase inhibitors, namely C $\bar{\text{I}}$ s inhibitor, α_2 -M, AT III and possibly α_1 -antiproteinase (Spragg and Austin, 1977; Donaldson and Harrison, 1982; van der Graaf, Koedam and Bouma, 1983).

The kinins produced are involved in the inflammatory response, contraction of smooth muscle, dilation of blood vessels, increased vascular permeability, leucocyte migration and production of the pain sensation (Abe, 1981). Kinins are vasoactive substances which might contribute, together with prostaglandins, to the regulation of blood pressure through peripheral arteriole dilation (Abe, 1981).

The kallikrein-kinin system is essential in the inflammatory response and is closely associated with the immune response, facilitating the movement of macrophages and lymphocytes to the site of tissue damage (Schorlemmer, 1977).

The biological effects exerted by this pathway involve a close relationship with Factor XII, as illustrated in Figure 2.8.1. Consequently, the coagulation, fibrinolytic and inflammatory mechanisms share a focal point through the activation of Factor XII. This further displays the inter-dependence of physiological pathways in the maintenance of haemostasis.

2.5 Plasma fibrinolytic system

The formation of a fibrin clot at the site of tissue damage results in the activation of the fibrinolytic pathway. Plasminogen, the zymogen of plasmin, is adsorbed onto the surface of the fibrin

network, together with activators that are present locally in the vessel wall or in the circulating blood (Moroz, 1980). Cells circulating in the plasma account for most of the fibrinolytic activity, having plasminogen associated with the cells surface (Todd, 1959; Moroz, 1980). Polymorphonuclear leucocytes are the major component of the cellular phase. They contain enzymes, such as cathespin G and elastase-like proteinase (Plow and Edington, 1978; Moroz, 1977) and possess surface receptors for fibrin (Sherman and Lee, 1977), capable of facilitating interaction with the substrate.

Polymorphonuclear leucocytes are observed in proximity to the endothelium in normal veins, and the presence of normal numbers of polymorphonuclear leucocytes protects against thrombosis (Stuart et al., 1977). Hence, these cells are functionally equipped, numerically adequate and appropriately situated for removal of physiological fibrin deposits.

The plasminogen molecule consists of two domains. One domain contains the active site whilst the other bears the binding region. The active form of plasminogen is called Glu-plasminogen due to the residue of glutamine at the N-terminus. Cleavage of Glu-plasminogen results in the release of a small peptide from the N-terminus producing the lysine-containing residue called Lys-plasminogen. This partial degradation enhances activation of the enzyme and increases its affinity for fibrin (Thorsen, 1975) and for α_2 -antiplasmin (Wimen, Lijnen and Collen, 1979). This is probably by exposure of the lysine-catalysing substrate binding site.

Plasminogen activators have proved difficult to isolate; however, Rijken et al. (1979) succeeded in the purification of an activator from

human uterine tissue. It was established through antibody binding studies that this activator was derived from tissue (Rijken, 1980). Therefore, if both plasminogen and its activators are adsorbed onto the surface of fibrin then the activation of plasminogen will occur only in the presence of fibrin.

The fibrinolytic system may be activated by venous occlusion, strenuous exercise, stress, certain drugs and natural damage. Regulation of this pathway is essential to prevent haemorrhaging. The most significant plasmin inhibitor is α_2 -antiplasmin; it binds plasmin in a 1:1 molar stoichiometric complex devoid of proteinase activity (Moroi and Aoki, 1976; Wimen and Collen, 1977). The α_2 -antiplasmin inhibits plasmin and in addition decreases the binding of Lys-plasminogen to fibrin (Aoki, Moroi and Tackiya, 1978). A fast-acting plasmin inhibitor has been purified from platelets and shows similarities to α_2 -antiplasmin both in structure and function (Sandbjerg-Hansen and Clemmensen, 1980).

Early attempts to characterise FDPs by immunoelectrophoresis established the similarity between degradation products X, Y, D and E formed in vitro (Fisher et al., 1967). More recently, immuno-absorption or physical methods have been used (Merskey et al., 1980).

FDPs are known to influence the immune system and it has been shown that plasmin-induced FDPs increase leucocyte chemotactic activity and vascular permeability (Sueishi, Nano and Tanaka, 1981). Results from FDP administration to mice indicate inhibition of the immune system but the exact mechanism has not yet been fully elucidated.

2.6 Platelets in blood coagulation

Platelets are small non-nuclear cells derived from megakaryocytes. In the quiescent state, platelets contain organelles such as dense granules, alpha granules, lysosomes, peroxidases and mitochondria (Zucker, 1980).

The dense granules contain calcium, adenosine 5'-diphosphate (ADP), adenosine 5'-triphosphate (ATP), 5-hydroxytryptamine and possibly α_1 -antiproteinases (Holmsen, 1975). The granules are considered to contain platelet-derived coagulation factors including fibrinogen, Factor V and platelet Factor VIII (Nurden and Caen, 1979; Zucker, Brockman and Kaplan, 1979).

Lysosomes make up only a small portion of platelet organelles but contain a number of degradative enzymes such as cathepsin (Brentfield and Bainton, 1975; Ehrlich and Gordon, 1976). These degradative enzymes may be important in platelet phagocytic reactions (Niewiarowski, 1977).

Platelets are essential for haemostasis in several ways as they adhere to sites of tissue damage and to each other, to form a haemostatic plug which helps restore the patency of the vessel. The platelet also provides the phospholipid required for some stages of the coagulation cascade, and finally the platelet releases presynthesised coagulation factors into the micro-environment, thus promoting clot formation.

Activation of platelets may occur via specific receptors on the platelet surface for collagen, thrombin and ADP causing viscous metamorphosis, with possibly the release of cellular components. Activation of platelets also results in exposure of receptor sites on the platelet surface, for example, for fibrinogen (Bennett, Valaire and Burch, 1981). Platelet

activation to form aggregates may also be associated with immune complex formation as would occur in some disease states (Clark et al., 1980).

Recent advances in platelet studies have suggested that platelets may undergo viscous metamorphosis without the release reaction occurring and that if these cells are left in vitro for one to two hours, the cells regain their normal shape (Kindness, personal communication). However, once the release reaction has occurred, the platelets disintegrate and are no longer able to reassociate (Mustard and Packham, 1970).

In the platelet aggregation, thrombin appears to act on platelets by association with platelet component glyocalin (Ganguly and Gould, 1979; Okumura, Hasitz and Jamieson, 1978). Several investigators have characterised thrombin binding to platelets noting high and low affinity binding (Martin et al., 1976; Tam and Detwiler, 1978). These differing affinities may represent two classes of thrombin platelet binding sites (Tam, Fenton and Detwiler, 1979).

Once membrane bound, thrombin potently induces platelet aggregation and secretion apparently by diverse mechanisms. When stirred with platelets, thrombin causes platelet aggregation and secretion of granule contents, while in the absence of stirring only secretion occurs (Mills and MacFarlane, 1976). At low concentrations of thrombin, platelet secretion may be mediated by prostaglandin endoperoxides and thromboxanes, while higher thrombin concentrations may elicit secretion even when cyclo-oxygenase is blocked and cyclic AMP levels are elevated (Charo, Feinman and Detwiler, 1977; Shuman, Botney and Fenton, 1979; Kindness, Long and Williamson, 1981). The biochemical

basis of this type of platelet activation is not known but could involve translocation of calcium across the membrane (Kinlough-Rathbone et al., 1977) or release of lipophospholipid from membranes (Chignard et al., 1979; Gerrard et al., 1979). There is some evidence to suggest that thrombin, perhaps generated on platelet surfaces, may be an important factor in the mediation of collagen-induced platelet aggregation in plasma (Nichols et al., 1980).

2.7 Plasma lipoproteins

Plasma lipoproteins are the "vehicle" of lipids in the blood stream. They are organised in such a way that they are able to carry water-insoluble lipids in an aqueous solution. The apolar lipid constituents of lipoproteins, triglyceride and esterified cholesterol, are organised in a core surrounded by a surface film made by cholesterol, phospholipid and proteins (Jackson, Morrisett and Gotto, 1976). The protein moiety of lipoprotein is heterogeneous and many different apoproteins have been identified (Schaefer et al., 1978). Lipoprotein synthesis takes place only in the intestine and liver. In particular, the liver synthesises very low density lipoprotein (VLDL) and high density lipoprotein (HDL); the intestine synthesises chylomicrons, VLDL and HDL (Eisenberg, 1976).

The catabolism of plasma lipoproteins takes place by three different mechanisms. Lipid and protein exchange and/or transport, enzyme modification of lipoproteins and cellular uptake by means of high affinity receptors and non specific endocytosis (Smith, Pavnall and Gotto, 1978).

The exchange and transfer of lipids among lipoprotein classes is well known; however, the mechanism and quantitative aspects are still

unclear (Smith, Pavnall and Gotto, 1978).

Lipoprotein lipase (LPL), hepatic lipase (HL) and lecithin cholesterol acyl transferase (LCAT) are recognised as the main effectors of the enzyme lipoprotein catabolism (Smith, Pavnall and Gotto, 1978). LPL catalyses the hydrolysis of the triglycerides of chylomicrons and the VLDL at the vascular endothelium and by its action the lipoproteins are transformed to lipoprotein remnants (Redgrave, 1970; Robinson, 1970). Chylomicron remnants are sequestered by the liver where they suppress cholesterol biosynthesis (Nervi and Dietschy, 1975). VLDL remnants may either be sequestered by the liver or peripheral tissues; however, under normal circumstances, VLDL remnants are further catabolised to LDL (Sigurdsson, Nicoll and Lewis, 1975).

LCAT is synthesised in the liver and secreted into blood where it catalyses the esterification of cholesterol and a fatty acid derived from phosphatidycholine. VLDL and HDL are LCAT substrates and LCAT is involved with the regulation of cholesterol exchange between lipoprotein and tissues (Glomset and Noruin, 1973).

Catabolism of lipoprotein by peripheral cells occurs by interaction of lipoproteins with specific receptor sites (Mahley and Innerarity, 1977). The action of LPL occurs at endothelial surfaces; it catalyses the hydrolysis of triglycerides of lipoproteins to form free fatty acids and glycerol.

2.8 Interrelationship of blood coagulation, kinin, complement and fibrinolytic pathways

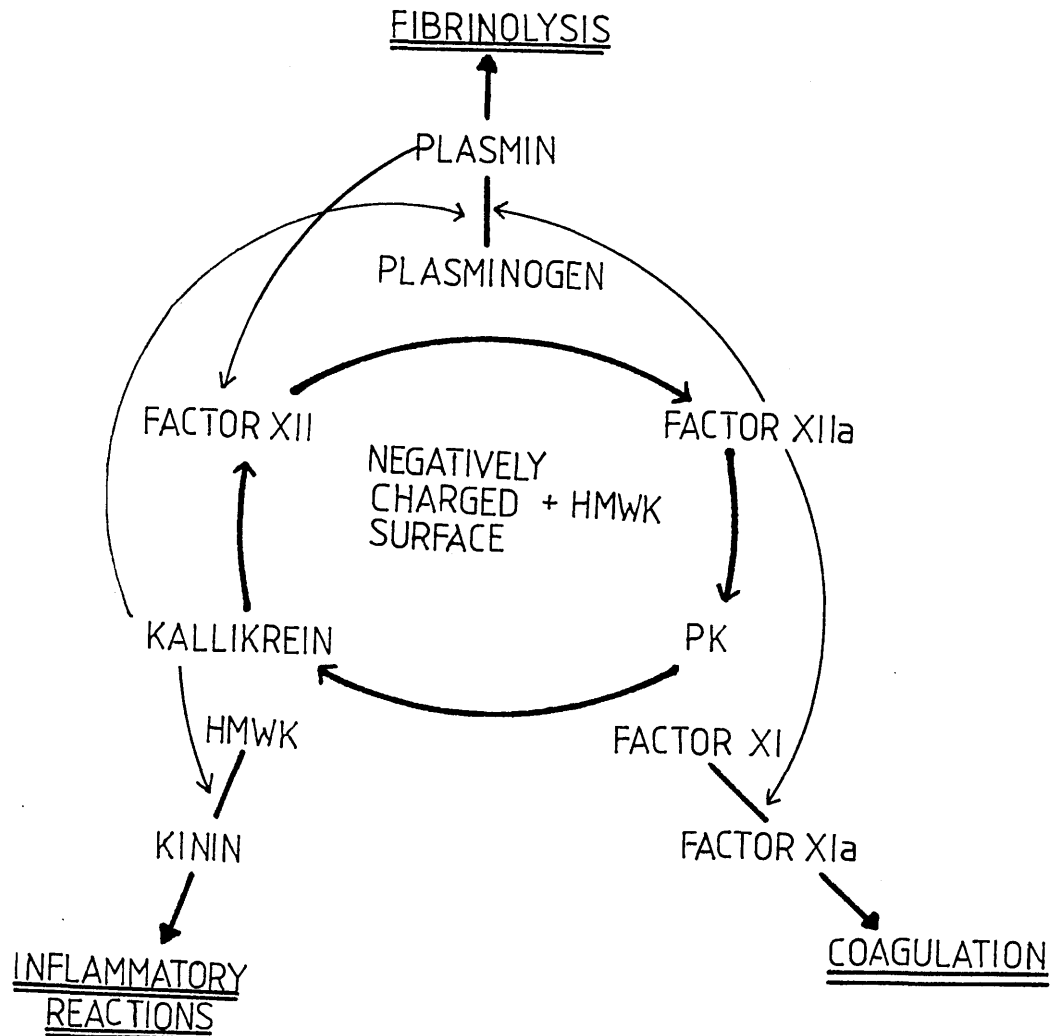
The coagulation, kinin, complement and fibrinolytic systems form an intricate defence mechanism against mechanical or biological invasion of the body. As each of these systems becomes more clearly understood,

it becomes apparent that they should not be regarded as separate systems but rather as interacting components of the overall defence mechanisms (Fig. 2.8.1).

Each system described previously is multi-component and involves the action of proteinases which are formed from circulating precursors. They also involve cascade sequences in which a small stimulus is amplified. Each system is partially controlled by a series of inhibitors which act either by destroying the activity of a component or neutralising one of the proteinases. Each of these attributes suggests close links to the coagulation mechanism. Examination of these systems suggests the presence of such links around the contact phase of blood coagulation, that is Factor XII, prekallikrein, HMWK and Factor XI.

The end product of this relationship is that a steady state of haemostasis is maintained with a fine balance between formation and break down of blood clots.

Inter-relationship of blood coagulation,
fibrinolysis and inflammatory reactions



3. THE IMMUNE SYSTEM

3.1 The Immune response

The essence of immunity lies in the ability to distinguish self from non-self. It is upon the ability to make this distinction that the organism has built a biological defence system geared to fend off pathological invaders. The diversity of the immune response is reflected in the apparent ability of the organism to recognise and respond to a large number of intrusive foreign antigens.

The functions of one area of the immune system, the humoral response, are conducted by a series of white blood cells, macrophages and lymphocytes. The latter cells are present in two forms: thymus-dependent (T) lymphocytes and thymus-independent, bone marrow-derived (B) lymphocytes. The cells of the humoral response work together, in ways that are not completely understood, to produce antibodies which are directed against the challenging antigen. These specific antibodies are released into the circulation by antibody-producing plasma cells.

The other aspect of immunity does not depend on secreted antibodies but rather on the direct action of antigen-specific lymphocytes. Cell-mediated immune responses rely on direct contact between target cells and effector lymphocytes, the latter being T cells (Katz, 1977).

Experiments, primarily by Glick, Chang and Japp (1956) and Cooper et al. (1966) on the chicken and by Millar (1962) on the mouse showed that not all lymphocytes within an individual were functionally equivalent. By removing a particular lymphoid organ early in the development of the host, and then challenging with an antigen, the immune system was examined. These experiments led to the view that depletion of chicken B lymphocytes by bursectomy resulted in the

inability to mount humoral immune responses (i.e. make antibody) without seriously affecting the cell-mediated immunity. Depletion of T lymphocytes by thymectomy resulted in the inability to mount both humoral and cell-mediated responses. The conclusions of these experiments were that T and B cells must interact before antibody could be produced in response to a specific antigen and that B cells were directly responsible for the synthesis and secretion of antibody, while T cells provided essential regulatory signals (Claman, Chaperon and Triplett, 1966).

A more refined picture of how T and B cells work to mount a humoral response came from experiments by Mitchison (1971). These experiments were designed to determine whether or not T and B cells recognised the same or different determinants on a complex antigen. This work used immunogenic carriers bearing specific haptens which were themselves antigenic but not immunogenic. To elicit an anti-hapten response they must be attached to an immunogenic substance. Thus, an injection of 2'-4'-dinitrophenol did not elicit a response but when attached to bovine serum albumin and injected, a response resulted. The antibodies produced then recognised and bound 2'-4'-dinitrophenol alone or when coupled to a number of other carriers. This "carrier" effect was a key piece of evidence implicating the recognition of two different determinants during the course of the humoral response. The critical experiment demonstrating that T cells recognised the carrier determinants was done by Raff (1970).

Each cell type associated with the immune response is now discussed in detail.

3.2 B cells

B cells are generally described as the antibody producing cells of the humoral immune response. These cells can be distinguished from T cells by a set of characteristic surface antigens and a distinct path of differentiation.

The membrane marker of the B lymphocyte is immunoglobulin. In 1900 Ehrlich predicted that B cells contained many thousand copies of the same immunoglobulin molecule which served as specific receptors for antigen and harbingers of the antibodies which their plasma cell progeny would ultimately secrete. These propositions were found to be correct by Moller (1961), and in 1970 Raff, Sternberg and Taylor demonstrated that immunoglobulin was found on some but not all lymphoid cells. They also found that any one cell produced only one immunoglobulin; whether the membrane immunoglobulin functions as an antigen receptor remains unanswered (Sato and Geffer, 1981).

Work by Ada and Byrt (1969) and Warner, Byrt and Ada (1970) established two facts in cellular immunology. Firstly, antigen reacts with specific lymphocytes capable of contributing to the immune response to that antigen only. Secondly, the reaction of antigen with specific B lymphocytes is through the receptor activity of their surface immunoglobulin. Expanding on these studies, Warner, Byrt and Ada (1970) determined that surface immunoglobulin acted as a vehicle for the antigen-lymphocyte interaction. While these experiments did not offer definitive proof that the B cell receptor for antigen was surface immunoglobulin, they were certainly suggestive of the importance of possible B cell-antigen interactions.

In 1974 Julius and Herzenberg used direct cell selection to show that in the course of an immune response to antigen X, antibodies produced late in the response bound antigen more tightly than antibodies which appeared earlier. This may be due to preferential selection of B cells bearing high avidity receptors under conditions where antigen concentration is decreasing. This avidity increase can be used as a monitor for the progress of the immune response.

Individual B cells differ in their expression of surface characteristics and also have been found to differ from one another somewhat in their ability to make an immune response (Saito and Geffer, 1981). One of the most important functional differences between individual B cells is their relative dependence upon T cells in the successful production of antibody. As stated previously, the humoral response to most antigens required both T and B cell participation. Studies with T cell-deprived mice have demonstrated that these immunodeficient animals are capable of producing antibodies to certain kinds of antigen, known as T-independent antigens (Scher et al., 1975). It is, however, not yet clear whether these groups of B cells represent different cell lineages or different stages of one cell lineage.

In the immune response, antigen binding results in the initiation of two distinct phenomena: proliferation of the appropriate B cell clones and differentiation of those antigen-binding cells into antibody-secreting cells. This was demonstrated by the work of Nossal and Ada (1971) in which animals immunised with an antigen were found to contain 100- to 1000-fold more antigen-binding cells specific for the antigen than naive animals. Also, antigen-binding cells were themselves incapable of antibody production. Thus, antigen encounter and a family of appropriate cell-cell interactions, are responsible for

expanding the population of lymphocytes reactive to the intruding antigen. They then induce lymphocyte maturation into plasma cells capable of delivering secreted antibodies into serum to inactivate the antigen.

3.3 T cells

The possibility that functionally distinct populations of T cells existed came from studies conducted by Cantor and Asofsky (1970, 1972) using antisera specific for lymphocyte antigens (Lyt 1, 2 and 3) believed to be expressed on the surface of T cells. Cantor and Boyse (1975) demonstrated that selective displays of Lyt antigens distinguished precursors of helper T cells (Lyt 1 and 2) from precursors of suppressor T cells (Lyt 2 and 3). A third population, bearing all three Lyt antigens, was designated as an immature population of T cells which had not been committed to either helper or suppressor activities.

It has been found that helper T cells are required to stimulate B cells to divide and to differentiate into antibody-secreting cells. For conventional antigens, both the helper T cell and the B cell are specific for the same antigen although not necessarily for the same determinants. In experimental systems using hapten-bound carriers, the helper T cell is specific for the carrier whilst the B cell is specific for the hapten. Successful activation of an antibody response also involves the participation of macrophages. The T suppressor cell may influence this intricate network of interacting cells by altering the ability of the helper T cell, B cells or macrophages to function (Gershon and Kondo, 1971). That is, suppression could result by preventing macrophages from "presenting" antigen to T helper cells; by inhibiting B or T helper cell proliferation or by differentiation; or by actively eliminating B or T helper cell clones. The

action of T suppressor cells causes the animal to become tolerant to invading organisms. However, this phenomenon was found to be antigen-specific in that the recipient animal could still respond to a closely related antigen.

Studies carried out by Tada et al. (1974) showed that suppression of antibody formation was carrier-specific, suggesting that suppression could be directed at the T helper cell. They showed that an active soluble factor could be obtained from sonicated spleen cells or thymocytes of animals injected in such a way as to produce suppressor T cells. This "factor" mimics exactly the action of the suppressor T cells, and if injected prior to antigen stimulation, prevents anti-hapten antibody formation. Taniguchi and Takuhisa (1980) reported that the "factor" is elaborated in response to the interaction of a specific antigen with a specific T cell. The target of the "factor" is not a specific T helper cell but appears to be yet another T cell, that is, it elaborates an antigen-non-specific "factor". It was the second of these "factors" which resulted in the final suppression of antibody formation. The antigen-non-specific end point of this chain of reactions illustrates how a mono-specific suppressor cell could suppress all helper activity.

To determine the target cell at the end of the suppressor chain, experiments were carried out to eliminate the suppressor T cell population (Herzenberg et al., 1976). This did not, however, result in immediate resumption of an antibody response. This was thought to be due to complete absence or total inactivation of the specific T helper cell population. In contrast, the B cell population in suppressed animals is perfectly functional and can be activated by presentation of appropriate active T helper cells.

In summary, suppression is brought about by a cascade of events which involves several cell types and soluble substances which ultimately lead to the prevention of antibody formation. Suppressor cells and antigen are at the start of the cascade, and the target cell may be the T helper cell.

3.4 Macrophages and antigen presentation

The involvement of macrophages in the immune system was observed when it was shown that immune responses were enhanced when antigens were taken up by phagocytic cells but depressed when these cells were removed (Unanue and Askonas, 1968). These results led to the theory that the antigen must be "processed" by macrophages to stimulate lymphocytes.

The term macrophage describes a wide variety of phagocytic cells, present in a number of different tissues. The cells contain many lysosomes and surface receptors for both immunoglobulin and the third component of complement. The macrophage acts as an intermediary between free antigen and T helper cells, being recognised by the expression of a gene product called Ia present on the macrophage surface, specific for an antigen. Thus the essential requirements for T cell binding to macrophages are the antigen and Ia gene product specific for the antigen.

It was demonstrated by Rosenthal and Shevack (1973) that free antigen without macrophages, or macrophages alone, are not capable of activating T cells. If, however, macrophages were exposed to antigen and then the antigen washed off, the macrophage would still elicit an immune response. They also showed that animals incapable

of responding to a particular non-specific antigen are defective in the ability of their macrophages to "present" that antigen to the T cells in an effective way.

Sprent (1980) showed the association of macrophages, T and B cells to be as follows: T helper cells which have receptors specific for the antigen in association with the Ia molecule on the appropriate macrophage will only bind to and activate B cells bearing the same Ia molecule, resulting in B cell proliferation and maturation to give active immunoglobulins against the antigen. Thus B cells and macrophages present similar determinants to a reactive T cell although the acquisition of the antigen has been specific for the B cell and non-specific for the macrophage.

Activated macrophages release a variety of factors (Gery and Waksman, 1972) including lymphocyte-activating factor, which induces the appropriate T cells (but not B cells) to proliferate. Macrophages are stimulated to produce this factor by lipopolysaccharides or phytohaemagglutinin. The resting T cells exposed to this factor demonstrate an increased responsiveness to T cell mitogens (stimulators) such as concanavalin A or phytohaemagglutinin.

The failure of macrophages to process and present antigen is, as previously described, associated with the inability of the organism to mount an immune response against that antigen. This may result in the appearance of T suppressor cells (Pierres and Germain, 1978). Such T suppressor cells are specific for the antigen and can prevent any further response to that antigen even if macrophages are present.

It has been shown that T suppressor cells can bind free antigen while T helper cells require macrophage-antigen presentation. The failure of macrophages to take up antigen results in an excess of

circulating antigen. The T suppressor cells would therefore also remain unresponsive. Suppressor cells are readily generated when either antigen is administered in a form inimical to macrophage uptake, or when antigen is present in excess. In contrast, an immune response is seen if insoluble complexes of antigen, such as would occur due to macromolecular complex formation between polyanion and polycations, are administered. The presence of adjuvants which "activate" macrophages further enhances the immune response (Saito and Geftter, 1981).

3.5 Immunosuppressive influence of carrageenan

Carrageenans are being widely used as modifiers of the immune response in various animals particularly mice (Rumjanek and Brent, 1978; Turner and Higginbotham, 1977; Thomson, 1978; Thomson, Fowler and Pugh-Humphreys, 1979; Fowler and Thomson, 1979; Sakemi, Kuroswa and Nomata, 1980; Kolb et al., 1981; Thomson et al., 1981). Carrageenans have been reported to be toxic to macrophages in vitro (Allison, Harrington and Birbeck, 1966; Yung and Cadkowicz, 1978), and in vivo (Gordon, MacIntyre and McMillan, 1977; Simon and Jones, 1979; Lee, 1981).

A macrophage population is required for non-self detection in, for instance, bone marrow transplants in irradiated animals. Studies on the influence of the three fractions of carrageenan indicated that carrageenan suppressed resistance to grafts when a carrageenan dose of 500 µg per irradiated mouse was injected intravenously several hours before or after transplantation (Yung and Cudkowicz, 1977). The authors found that λ- and ι-carrageenan were more effective than κ-carrageenan, and that suppression was enhanced when irradiation

preceded transplantation by 24 hours.

Although the detailed mechanism of carrageenan-induced toxicity of macrophages is still to be elucidated, carrageenan is known to be readily taken up by macrophages, causing swelling and rupture of cells and apparently resulting in cell death since macrophages cannot readily degrade carrageenan (Ishizaka et al., 1980).

Carrageenans have been shown to act on lymphocytes, especially B cells, resulting in polyclonal antibody production, although previous studies suggested that carrageenans act only on macrophages and not on lymphocytes (Ishizaka et al., 1980; Cateinzaro, Schwartz and Graham, 1971). These differences may simply be due to different types of carrageenan having different biological activities.

Macrophage activity in the induction of inflammation is well documented and λ -carrageenan was shown to have the greatest inflammatory activity (Davies and Allison, 1976). Other work (Ishizaka et al., 1980) indicated, however, that there was no difference in polyclonal antibody production elicited by the three different carrageenan fractions assayed.

Carrageenans have been used together with other immunosuppressive drugs to prolong renal allograft survival in dogs (Calne, Wall and Wilkins, 1975). λ -Carrageenan was similarly found to be the most immunosuppressive of the fractions when given to rats which received heart transplants (Everson, Stacey and Bell, 1977). These effects were thought to be mediated through macrophage toxicity induced by the carrageenan. Mortality in these rats was high. However, the remaining rats had a high transplant survival time. Mortality may have been due to many factors such as bleeding from the operation site, disseminated intravascular coagulation and diverse effects of carrageenan on the blood.

4. THE RODENT SYSTEM

4.1 The rat

Within this study the rat was chosen as the experimental model to investigate Factor XII-related responses including coagulation, fibrinolysis and inflammation. The activation of the early stages of blood coagulation per se not only involve the above interrelated pathways but result in stimulation of platelet responses which have profound effects on blood flow. Similarly, the dissolution of a blood clot through the action of the enzyme plasmin yielding fibrinogen degradation products which are known to participate in immune responses (Girman et al., 1976).

The rat model has been widely used in the study of allograft rejection (Everson, Stacey and Bell, 1977), inflammation (Schwartz and Kellermeyer, 1969) and immune responsiveness (Wilson et al., 1978). Consequently this model affords both ex vivo and in vitro testing facilities with which the Factor XII-related pathways may be explored and perturbed using animal groups yielding statistically significant results both per animal and for total group size. In conclusion, the rodent system offers an ideal opportunity through which these complex pathways may be overviewed in an attempt to gain an understanding of the physiological effects of carrageenan on these test systems.

Objectives of the study

The objectives of this thesis may be defined under the following:-

1. To study the effect of carrageenan on Factor XII-mediated responses.

In particular, the interaction of these polyelectrolytes with:

- a) Factor XII-mediated blood coagulation;
- b) Factor XII-dependent fibrinolytic activity;
- c) Factor XII-prekallikrein activity.

The above responses are to be monitored using both amidolytic and functional techniques.

2. To investigate carrageenan-platelet effects and their possible role in vitro and ex vivo with respect to the responses outlined above.

3. Lipoproteins have been directly related to the onset of atherosclerosis and as such a study of carrageenan effects on lipoproteins, separated by density centrifugation, was carried out ex vivo.

4. Carrageenans have been noted to be immunosuppressive. The production of fibrinogen degradation products have also been found to modify immune responses. Consequently, any Factor XII-mediated response as generated or inhibited by carrageenan may directly affect immune reactions.

In summary, to the author's knowledge an integrated study of in vitro and ex vivo carrageenan effects using a single rat model has not been undertaken.

III MATERIALS AND METHODS

Polysaccharides

Carrageenans

Carrageenans were a gift from Mr. J. Pederson, Pectin Factory, Copenhagen, Denmark, and chemical analyses are detailed (Table 1, p. 50).

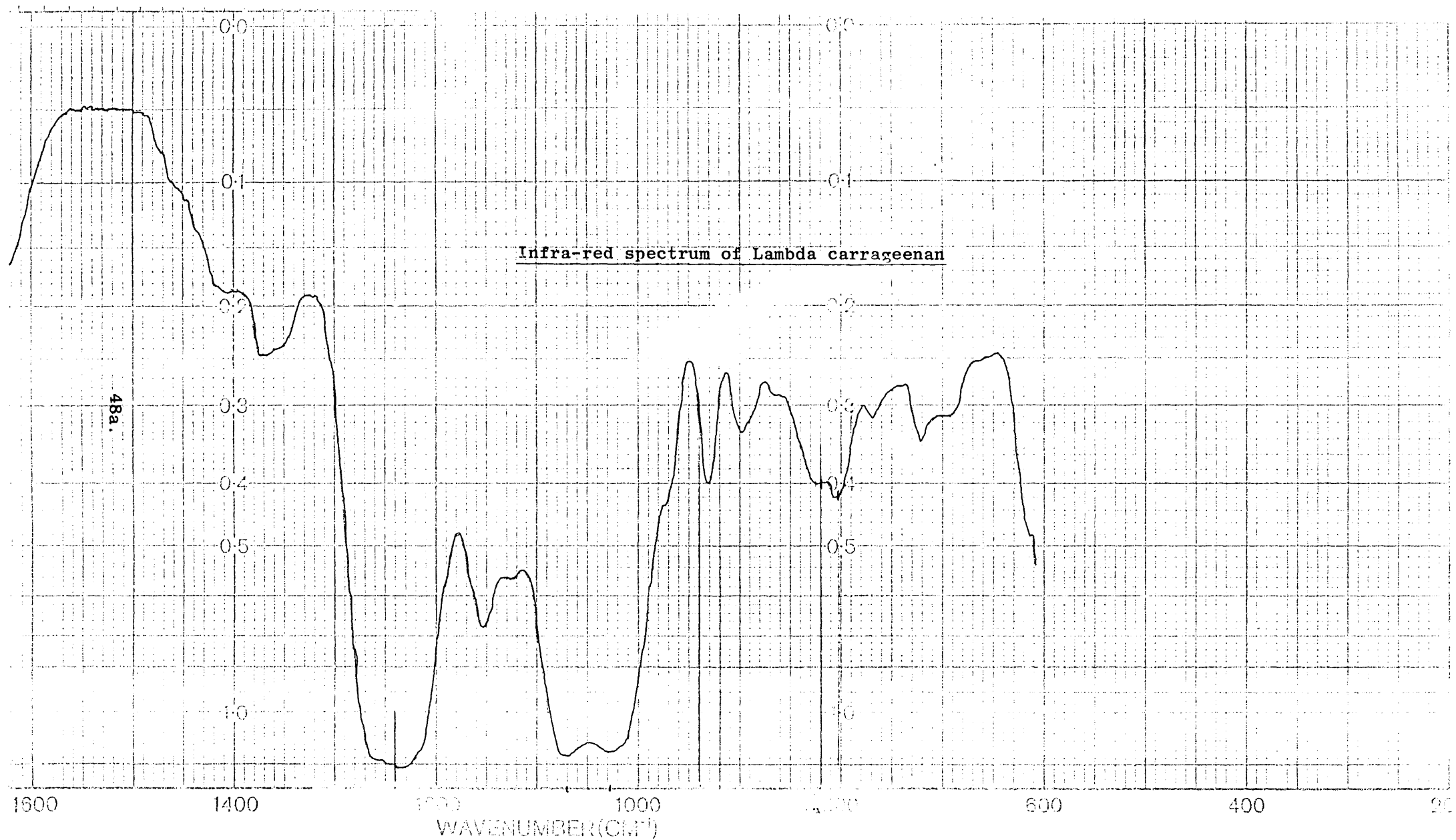
Infra-red analysis was carried out for each polysaccharide by J. Pederson and the spectra for the λ - and κ -carrageenans were found to be typical for these types of polysaccharide (J. Pederson, personal communication). The λ -carrageenan isolation process involved full alkali modification (J. Peterson, personal communication) as the spectra on page 48a illustrates (Rees, 1963; Percival, 1972). Subsequently this carrageenan will be referred to as alkali-modified λ -carrageenan throughout the text.

The salt forms of carrageenan used in this study reflect the method of isolation and purification as stated by the manufacturer.

Lyophilised samples were reconstituted in saline at a concentration of 10 mg ml^{-1} , pH 7.4. To aid dispersion the samples were heated in a 60°C water bath for 5 minutes. Prior to addition to plasma or injection, samples were further dispersed by vortexing for 30 seconds.

Glycosaminoglycan

Heparin (Porcine intestinal mucosa, grade 1, Lot No. 46C-0035) was obtained from Sigma Chemical Company Ltd., Poole, Dorset, U.K. The sodium salt form of heparin was found to contain 31.2% sulphate (w/w) ester.



Lambda fraction (λ)

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2

Heparin was dissolved in saline, pH 7.4 at a concentration of 10 mg ml⁻¹.

TABLE 1

Chemical analysis of lambda, iota and kappa carrageenans

Carrageenan	Salt Form	No.	Sulphate %*	Calcium %*	Potassium %*	Sodium %*
Lambda (λ)	Potassium	022100	29.94	0.29	10.04	0.14
Iota (ι)	Sodium	32460	32.10	0.47	5.75	2.34
	Calcium	32510	34.2	1.74	5.58	1.46
Kappa (κ)	Calcium	52731	22.9	0.85	5.25	0.65
	Potassium	52405	22.0	0.28	7.02	0.55

*Calculated on a weight to weight basis.

Preparation of buffers and general reagents

Acetate buffer pH 4.0

Sodium acetate 0.06M

pH adjusted to 4.0 with 1M acetic acid

Acetate buffer pH 5.7

Sodium acetate 0.015M

pH adjusted to 5.7 with 1M acetic acid

Phosphate buffer pH 7.4

Trisodium orthophosphate 0.1M

pH adjusted to 7.4 with 1M HCl

Phosphate-buffered saline pH 7.2

Monobasic sodium phosphate 0.036M

Dibasic sodium phosphate 0.065M

Sodium chloride 0.150M

Sodium azide 0.0015M

pH adjusted to 7.2 with 1M HCl

Tris (hydroxymethyl) methyl amine-HCl buffer pH 8.0

Tris (hydroxymethyl) methyl amine 0.1M

HCl 0.1M

To Tris (50 mls) add HCl (26.8 ml) then make up to

200 ml with distilled water

pH adjusted to 8.0 with 1M NaOH

Tris (hydroxymethyl) methyl amine-HCl buffer pH 7.8

Tris (hydroxymethyl) methyl amine	0.05M
Sodium chloride	0.012M
pH adjusted to 7.8 with 1M HCl	

Carbonate-bicarbonate buffer pH 9.6

Sodium carbonate	0.02M
Sodium bicarbonate	0.027M
Sodium azide	0.0015M
pH adjusted to 9.6 with 1M NaOH	

Veronal-buffered saline - Triton X 100

One veronal buffer saline tablet (Sigma per) 100 ml water	
Triton X 100	0.01% w/v

Glycine-hydroxide buffer pH 10.4

Glycine (50 mls)	0.2M
Sodium hydroxide (38.6 mls)	0.2M
Magnesium chloride	0.001M
Zinc chloride	0.001M
Made up to a final volume of 200 mls with distilled water	
pH adjusted to 10.4 with 1M NaOH	

Cholesterol acid reagent

Glacial acetic acid	200 mls
Acetic anhydride (concentrated)	210 mls
Sulphuric acid (concentrated)	30 mls

Ethanollic potassium hydroxide

Potassium hydroxide in absolute ethanol 0.35M

Sodium hydroxide in water pH 12.8 1.5M

Calcium chloride dihydrate in distilled water pH 7.4 0.25M

Saline pH 7.4

Sodium chloride in distilled water 0.15M

Anticoagulant solution pH 7.4

Trisodium citrate dihydrate in distilled water 3.8% w/v

General Reagents

Adenosine 5-diphosphate, ϵ -aminocaproic acid, Polybrene, (hexadimethrine bromide), flufenamic acid, Russell's viper venom, rat Immunoglobulin G, anti-rat Immunoglobulin G, 104 phosphate substrate, gluteraldehyde, alkaline phosphatase (Type VIIIs), Cephotest and Tris-Trisma base were obtained from Sigma Chemical Company Ltd., Poole, Dorset, U.K.

Bovine topical thrombin (Batch No. AC657) was obtained from Parke-Davis and Company, Detroit, Michigan, U.S.A. The lyophilised material was dissolved in the diluent provided, then further diluted to a concentration of 100 NIH U ml⁻¹ with saline. Samples were stored in sealed plastic tubes at -20°C until required, then diluted to the required strength with saline and kept on ice and used within 1 hour.

Human fibrinogen was obtained from KabiVitrum, Ealing, London, U.K. Fibrinogen was reconstituted with distilled water to give 10g l⁻¹ and stored in aliquots (2 ml) in sealed plastic tubes at 20°C until required.

Amidolytic substrates were obtained from Flow Laboratories, Irvine, Ayrshire, U.K. Amidolytic substrates: Bz-Ile-Glu-Gly-Arg-pNA (S-2222) and H-D-Pro-Phe-Arg-pNA (S-2302) were dissolved in distilled water at a concentration of 4 mM and stored in a dark bottle at 4°C. Amidolytic substrate H-D-Phe-Pip-Arg-pNA (S-2238) was dissolved in distilled water to give a concentration of 0.75 mM and stored as above. Amidolytic substrate H-D-Val-Lau-Lys-pNA (S-2251) was dissolved in distilled water to a concentration of 1.0 mM and stored as above.

Platelin (rabbit brain phospholipid) was obtained from General Diagnostics, Warner Bros., Eastleigh, Hants., U.K. The lyophilised reagent was dissolved in distilled water as directed by the manufacturer, then further diluted 1:2 with saline and kept on ice until required.

Heparin-Sepharose 4B was obtained from Pharmacia Fine Chemicals, Hounslow, Middlesex, U.K. The gel (5g) was swollen and equilibrated using phosphate buffer (0.1M, pH 7.4).

Polystyrene beads (3.2 mm diameter) were obtained from Euromatic Ltd., Brentford, Middlesex, U.K.

Sheep red blood cells and Minimal Essential Media were obtained from Gibco Ltd., Paisley, U.K.

Antisera specific for rat or mouse: IgG and IgM were purchased from Miles Laboratories, Stoke Poges, Slough, U.K.

Stock solutions of thrombin and fibrinogen stored at -20°C were routinely tested for clotting activity by coagulation assay tests on pooled control plasma.

All other reagents used were obtained from Sigma Chemicals Ltd., Poole, Dorset, U.K. and were of Analar grade unless otherwise stated.

Methods

Route of polysaccharide administration

Solutions of carrageenans (10 mg ml^{-1}) were prepared in saline and injected into the intraperitoneal cavity, according to rat weight, using a graduated polypropylene syringe (1.0 ml) and $20 \times 1\frac{1}{2}$ g needle.

In order to determine a zero hour response the animal was bled immediately, under anaesthetic, by cardiac puncture. For subsequent sampling the rat was allowed to recover before being returned to a cage, then subsequently anaesthetised and bled by cardiac puncture. Repeated anaesthesia was found to have no significant effects on the experimental results.

Treatment of experimental results

Results obtained were expressed as the mean and standard error where the number of readings (n) was no less than three for in vitro studies and $n > 5$ for ex vivo studies. In some cases standard error bars lay within the dimensions of the symbol.

For each assay the control values were obtained and are presented together with the number of readings per point. Where clotting time exceeded the control value by an arbitrary factor of 5, a clotting time of infinity (∞) was recorded. Activities in ex vivo assays were expressed in relation to similar activities recorded in control saline-treated animals used over the same time course as the test animals.

Preparation of plasma

Platelet-rich plasma

Rats were anaesthetised, then bled by cardiac puncture into a polypropylene syringe (10 ml) containing 3.8% w/v trisodium citrate (9 volumes of blood to 1 volume citrate) using a 20g x 1½ gauge needle. Whole citrated blood was centrifuged at 200 x g for 10 minutes at ambient temperature (25°C). The red cell-free platelet suspension was transferred to a plastic tube and used immediately. Pooled plasma

was obtained from not less than 4 animals. Microscopic analysis was carried out to confirm that a red cell free preparation was obtained.

Platelet-poor plasma

Following removal of platelet-rich plasma, the remaining blood was further clarified by centrifugation at 750 x g for 10 minutes at ambient temperature. The supernatant was transferred to a plastic tube and, unless stated, used immediately. A plasma pool from no less than 4 animals was used.

For ex vivo studies the animals were injected intraperitoneally with polysaccharide according to animal weight, then plasma prepared as above. Samples were kept separate in this instance and immediately assayed.

Preparation of modified plasma

The use of plasma depleted of proteinases or proteinase inhibitors allowed comparison of polysaccharide-protein interaction in the presence and absence of proteins associated with the coagulation pathway.

Factor XII-depleted plasma

The depletion of Factor XII from normal pooled rat plasma was carried out using the method of Nossel (1966). To pooled rat plasma (4 ml) in a polyethylene tube was added Whatman Celite 512 (20 mg ml⁻¹). The tube was sealed and incubated at 37°C for 70 minutes, with frequent mixing of the contents during the course of the incubation. The preparation was centrifuged at 2,500 x g for 10 minutes at 4°C. The supernatant was removed and dispensed into a plastic tube and

maintained at 37°C for 18 hours prior to use.

Thrombin clotting time, Russell's viper venom clotting time and partial thromboplastin clotting time were carried out on plasma sample both in the presence and absence of polysaccharide.

Antithrombin III-depleted plasma

Antithrombin III (AT III) was depleted from normal rat plasma by the method of Barrowcliffe et al. (1978). Plasma was eluted through a column containing Heparin-Sepharose 4B by the following procedure.

To a 5g sample of freeze-dried Heparin-Sepharose 4B (Pharmacia Chemicals) was added phosphate buffer (0.1 M pH 7.4) in a glass filter; the Heparin-Sepharose was then washed with the buffer (150 ml). The gel (30 ml) was packed into a plastic column (Pharmacia Chemicals) and equilibrated with 20 washes of phosphate buffer (0.1 M pH 7.4) until the absorbance of the eluant at 280 nm fell below 0.05. All procedures were carried out at 4°C.

Excess plasma was applied to the column at a flow rate of 1.0 ml minute⁻¹. Once plasma started to elute, it was continuously passed through the column for three hours. The AT III plasma was then collected in plastic tubes and used immediately. AT III was eluted from the column using a sodium chloride gradient of 0-1.5M in phosphate buffer (0.1M pH 7.4).

Preparation of serum

Rats were anaesthetised and bled by cardiac puncture into a polypropylene syringe (10 ml) using a 20g x 1½ gauge needle. The uncitrated blood was transferred to glass tubes and left at room

temperature for 2 hours to allow clot retraction. The clot formed was removed and the supernatant centrifuged at 750 x g for 5 minutes. The resultant cell-free serum was used immediately.

1. COAGULATION STUDIES

1.1 Clotting assays

Standard procedures for clotting were used in the study of carrageenan effects on the intrinsic pathway of blood coagulation. The galactan sulphate effects on whole blood, Factor XII, Factor X and thrombin clotting time were investigated. Carrageenan effects on plasma systems both in the presence and absence of proteinases and their inhibitors were examined in order to elucidate potential interactions of macroions. Amidolytic assays were used, where appropriate, in support of the functional clotting technique to further highlight points of interaction in the coagulation cascade.

1.1.1 Whole blood clotting assay

The method was modified from that of Lee and White (1913) and carried out in a plastic test system. Carrageenan was prepared in saline to give a final concentration of 1.0 mg ml^{-1} and further dilutions were carried out in saline.

Whole citrated blood was obtained from non-treated animals. Saline or carrageenan ($100 \mu\text{l}$) was added to whole citrated blood ($200 \mu\text{l}$) in plastic tubes. Mixing was ensured by vortex mixing for 15 seconds before placing the tubes in a water bath (37°C). Calcium chloride (0.025M ; $100 \mu\text{l}$) was added and mixed by vortex for 15 seconds. The tubes were incubated in the water bath and tilted through an 180° angle every minute until a clot was observed. The time taken for each clot to form was noted and the arithmetic mean of triplicate experiments was obtained.

The coagulation cascade is known to be activated when the blood (plasma) comes into contact with negatively charged surfaces such as glass or kaolin. Such surfaces were avoided in the following procedure so that the effects of polysaccharides themselves could be ascertained.

If no clot formed in the tube containing polysaccharide after 1000 seconds, a clotting time of greater than 1000 seconds was recorded.

1.1.2 Partial thromboplastin clotting time assay

The method used was a modified form of the method of Langdell and Wagner (1955) using a plastic system and an automatic coagulometer.

Normal rat platelet-poor plasma from four male rats was obtained and pooled. Carrageenans and heparin were dissolved to a concentration of 1.0 mg ml^{-1} in saline and diluted further in saline. Platelin was prepared according to the instructions given by the manufacturer then further diluted 1:2 with saline.

Carrageenan, heparin or saline (100 μl) was added to plasma (100 μl) in a plastic tube. The mixture was incubated at 37°C for 120 seconds prior to the addition of platelin. After 60 seconds incubation, calcium chloride (0.025M, 100 μl) was added and the automatic timer started. The clotting time was taken as the mean of triplicate readings.

If a clot in the presence of polysaccharide had not formed after 300 seconds, a clotting time of greater than 300 seconds was recorded. Control readings in the absence of carrageenan were in the range 40-55 seconds.

The above method was also used in plasma samples depleted of Factor XII by celite exhaustion.

For ex vivo assays, plasma was obtained from polysaccharide-injected animals. To plasma (100 μ l) was added platelin (100 μ l) and the solution mixed and incubated for 60 seconds at 37°C before the addition of calcium chloride (0.025M, 100 μ l).

Triplicate readings were obtained per animal with no less than five animals per group.

1.1.3 Russell's viper venom clotting assay

A modification of the method of Furie et al (1974) was used to study the effects of polysaccharides on Factor X activation. Plasma from six male rats was pooled. Russell's viper venom was dissolved in saline to give 1×10^{-4} g ml⁻¹ and mixed 1:16 (v/v) with platelin (Platelin/RVV).

Plasma (100 μ l) in plastic tubes was incubated with polysaccharide or saline (100 μ l) for 120 seconds at 37°C. Platelin/RVV (100 μ l) was added and mixed, then incubated at 37°C for 30 seconds. Calcium chloride (0.025M, 100 μ l) was added and the clotting time recorded. The mean of triplicate readings was recorded. A reading of greater than 300 seconds was recorded. This method was repeated for Factor XII-depleted and AT III-depleted plasma.

For ex vivo studies, plasma was collected from animals which had previously been injected with polysaccharide and the assay carried out as detailed below.

To plasma (100 μ l) was added Platelin/RVV (100 μ l) and the mixture incubated at 37°C for 30 seconds prior to the addition of calcium chloride (0.025M, 100 μ l). Triplicate readings per animal were recorded.

1.1.4 Thrombin clotting time assay

Thrombin clotting time was assayed by a modification of the method of Ratnoff (1954). Plasma (100 μ l) was incubated with carra-geenan (100 μ l) in a plastic tube for 120 seconds at 37°C. Thrombin (25 NIH U ml⁻¹, 100 μ l) was added and the clotting time recorded. The mean of triplicate readings was recorded.

This method was also carried out for Factor XII-depleted and AT III-depleted plasma.

For ex vivo assays, plasma (100 μ l) from injected animals was incubated with saline (100 μ l) for 120 seconds prior to addition of thrombin (25 NIH U ml⁻¹, 100 μ l). The average of triplicate readings per animal was recorded.

1.2 Amidolytic assays

1.2.1 Determination of Factor X in plasma

Factor X activity in rat plasma was determined using the method of Aurell et al. (1977).

Plasma was diluted 1:25 (v/v) with Tris-NaCl buffer pH 8.0 (Tris 0.05M; NaCl 0.227M). Polysaccharide (1.0 mg - 1.0 μ g ml⁻¹), heparin (1.0 mg - 1.0 g ml⁻¹) or saline (50 μ l) was added to the diluted plasma (200 μ l) and incubated for 180 seconds prior to the addition of 50 μ l of a 2:1 (v/v) mixture of Russell's viper venom (0.1 mg ml⁻¹) and calcium chloride (0.4M). The solution was incubated for a further 60 seconds at 37°C before addition of substrate S2222 (0.008M; 200 μ l). Acetic acid (50% v/v; 300 μ l) was added after 15 minutes to stop further reaction. Interaction of the enzyme with the chromogenic substrate resulted in the release of a chromophore

paranitroaniline, the absorbance at 405 nm of which was measured spectrophotometrically.

Ex vivo assays were carried out using plasma obtained from polysaccharide-injected animals. To diluted plasma (200 μ l) was added Russell's viper venom and calcium chloride as above. After incubation for 60 seconds at 37°C, substrate was added and the reaction terminated after 15 minutes by addition of acetic acid (300 μ l). Spectrophotometric readings were obtained as detailed above.

1.2.2 Determination of thrombin activity in plasma

Thrombin activity was assayed by the method of Ødegard (1975). Plasma was diluted 1:61 (v/v) with Tris buffer (0.05M) pH 8.4 containing disodium ethylene diamine tetra acetic acid (0.0075M) and sodium chloride (0.175M).

To diluted plasma (200 μ l), polysaccharide (1.0 mg - 1.0 μ g ml⁻¹), heparin (1.0 mg - 1.0 μ g ml⁻¹) or saline (50 μ l) was added and incubated for 180 seconds prior to the addition of thrombin (10 NIH U ml⁻¹; 50 μ l). Following incubation for 30 seconds, a mixture containing substrate S2251 (0.5M) and Polybrene (0.34 mg ml⁻¹) was added (150 μ l) and incubation continued for 30 seconds. The reaction was stopped by the addition of acetic acid (50%, 300 μ l).

The ex vivo assays were carried out using plasma from injected animals. To diluted plasma (200 μ l), thrombin was added (50 μ l); then the mixture was incubated for 30 seconds prior to the addition of the substrate-Polybrene mixture (150 μ l). Amidolysis was stopped after 30 seconds by the addition of acetic acid (300 μ l) and the samples assayed as detailed previously.

2. FIBRINOLYSIS

Enzymic cleavage of Factor XII not only results in the initiation of the coagulation cascade but also activation of the fibrinolytic and kallikrein pathways. The fibrinolytic and kallikrein systems were studied to further investigate the possible sites of polysaccharide-plasma proteinase.

2.1 Euglobulin precipitation of plasma

Preparation of the euglobulin fraction of plasma was performed using a modification of the method of Kluft, Brakman and Veldhuyzen-Stolk (1976).

To a polycarbonate tube was added plasma (1.0 ml) and polysaccharide ($1 \text{ mg} - 1 \mu\text{g ml}^{-1}$) or saline. The preparations were mixed and incubated at 37°C for 10 minutes prior to the addition of glacial acetic acid (0.017% v/v; 9 ml). The tubes were sealed, and then the contents were thoroughly mixed and maintained at 4°C for 2 hours. The tubes were centrifuged at $750 \times g$ for 10 minutes at 4°C and the supernatant discarded. The tubes were inverted and, in order to remove residual supernatant, dried with a cone of filter paper (Whatman No. 1). The euglobulin pellet was resuspended in Tris HCl buffer pH 7.8 (1.0 ml).

2.2 Inhibition studies

The mechanism of carrageenan-induced fibrinolytic activity was investigated by experiments designed to study plasminogen-plasmin induced fibrinolysis. Initially ϵ -ACA (2 mM), a plasminogen inhibitor was added to plasma and incubated for 3 minutes

at 37°C prior to euglobulin precipitation. Secondly, flufenamic acid (12 mM), an inhibitor of plasmin production via an influence on an inhibitor of the first component of the complement pathway, was incorporated into the plasma and treated as above.

Ex vivo studies involved the use of plasma from polysaccharide-injected animals, and the incorporation of saline, ϵ -ACA or flufenamic acid prior to euglobulin precipitation.

2.3 Preparation of fibrin plates

Stock fibrinogen (10 g l⁻¹) was prepared in distilled water and stored in plastic tubes at -20°C. Thrombin was prepared at a concentration of 100 NIH U ml⁻¹ and similarly stored at -20°C.

Petri dishes were marked into quadrants, and a filter paper attached to the inner surface of the lid. The paper was moistened in order to prevent drying of the plate.

Stock fibrinogen was further diluted with Tris HCl pH 7.8 and aliquots (2 ml) were put into plastic tubes along with Tris HCl buffer pH 7.8 (7 - 8 mls). Thrombin (10 NIH U ml⁻¹, 200 μ l) was added and the tube inverted, care being taken to avoid the formation of bubbles. The resultant solution was carefully poured in the Petri dish, care again being taken to avoid the formation of bubbles, and the contents allowed to set evenly in the dish. Plates were then left at 37°C for one hour, and euglobulin test solutions (30 μ l) were added to plates within two hours of preparation. The plates were incubated at 37°C for 18 hours after which they were inspected for areas of lysis. The area of lysis was calculated as the product of two perpendicular axes with the results expressed in mm². The mean and standard errors of duplicate readings were then presented graphically.

3. KININ SYSTEM

3.1 Determination of prekallikrein in plasma

Prekallikrein activation in rat plasma was measured by the method by Claesson et al. (1978). Plasma was diluted 1:12 (v/v) with Tris buffer (0.05M) pH 7.8 containing sodium chloride (0.012M).

Cephotest, diluted 1:10 (v/v) with buffer pH 7.8 was used as an activator. In a plastic tube Cephotest (400 μ l) was added to diluted plasma. The mixture was then incubated for 180 seconds with polysaccharide (1.0 mg - 1.0 μ g ml⁻¹), heparin (1.0 mg - 1.0 μ g ml⁻¹) or saline (50 μ l). Following incubation for 120 seconds, substrate S2302 (4 mM) was added (100 μ l) and the mixture further incubated for 60 seconds. The reaction was stopped by the addition of acetic acid (50% v/v, 300 μ l).

In all assays, reagent concentrations quoted are final concentrations in the reaction mix prior to acetic acid addition. Appropriate control tubes containing separately no polysaccharide, heparin, enzyme or substrate were included.

Paranitroaniline (pNA) released from substrates was determined spectrophotometrically at 405 nm using an SP 1800 spectrophotometer. Absorbance measured in control tubes was in the range 0.5 - 0.6.

4. PLATELET STUDIES

4.1 Platelet studies using platelet-rich plasma

Platelet-rich plasma (PRP) was prepared as previously described. A platelet count was determined by the addition of PRP (100 μ l) to ammonium oxalate (1% w/v, 1900 μ l). The mixture was allowed to stand for 20 minutes at ambient temperature before cells were counted with an improved Neubauer cell counting chamber and using phase contrast microscopy. Cell counts were always adjusted to a range of 2.4 - 3.5 $\times 10^5$ platelets mm^{-3} .

4.2 Platelet aggregation

Platelet aggregation studies were carried out using a modification of the method of Born (1962).

A single channel aggregometer (Malin Electronics, Ayr, U.K.) and a single channel recorder (Linseis GMBH, Germany) were used. The aggregometer and recorder were calibrated with PRP (0% light transmission) and PPR (100% light transmission) as arbitrary values for each plasma sample.

To PRP (140 μ l) was added polysaccharide (1 mg - 1 μ g ml^{-1}) or saline (10 μ l). The solution was stirred at 900 rpm at 37°C for 3 minutes before the addition of thrombin (10 μ l) or ADP (10 μ l) and aggregation was observed. The test volume of the reaction mix within the cuvette was always 160 μ l.

Ex vivo assays for platelet aggregation involved plasma (140 μ l) from polysaccharide-injected animals. The plasma was treated with ADP (50, 10, 1.0 mM; 10 μ l) and thrombin (1.5, 1.0, 0.5 NIH U ml^{-1} , 10 μ l).

4.3 Platelet adhesiveness

Assays of platelet adhesiveness were performed using the method of Morris et al. (1978). To saline-washed glass beads (100 microns, 250 mg) was added platelet-rich plasma (250 μ l) from carrageenan-treated animals. The tubes were rotated through 360° for 1 minute then left at ambient temperature for one hour to allow the beads to settle.

The supernatant (50 μ l) was removed into a dye solution containing 1:1 (v/v) mix of 20% (w/v) urea (to lyse red cells) to 1% bromophenol blue (to stain platelets) (1.0 ml). A sample was then removed and transferred to a haemocytometer and the platelets counted.

Percentage adhesiveness was calculated as:

$$\frac{\text{No. of platelets in control sample} - \text{No. of platelets in test sample}}{\text{No. of platelets in control sample}} \times 100$$

5. IMMUNOGLOBULIN STUDIES

5.1 Enzyme-linked immunosorbent assay for rat Immunoglobulin G

Animals were injected and serum samples collected as described previously (p. 58).

Preparation of crude globulin

Anti-rat IgG (sheep) was precipitated from solution with 2 volumes of disodium sulphate (27% w/v) and the mixture left at room temperature for 15 minutes. The mixture was centrifuged at 1200 x g for 10 minutes and the precipitate obtained resuspended in saline (2 ml). In order to remove excess sulphate the suspension was dialysed against buffered saline (PBS) for 12 hours at 4°C.

Estimation of protein concentration

The protein content of the globulin was determined spectrophotometrically at 280 nm.

Conjugation of alkaline phosphatase

Conjugation was carried out as outlined by Parratt, Nielson and White (1977). Alkaline phosphatase was combined with anti-rat IgG to a ratio of 3:1 (w/w). The alkaline phosphatase suspension was centrifuged (750 x g, 10 minutes, 4°C) and the supernatant discarded. The pellet was resuspended in the required volume of anti-rat IgG. The mixture was then dialysed against PBS pH 7.4 for 48 hours with two changes of PBS. Following dialysis, the mixture was transferred to a plastic tube and the volume made up to 1.25 ml with PBS.

Gluteraldehyde required for optimal conjugation of enzyme and antibody was added to a final concentration of 0.2% v/v. The mixture

was left at ambient temperature for 2-4 hours until a pale yellow colour developed. Finally, the solution was dialysed against 3 changes of PBS pH 7.4 (2 l) and one change of Tris buffer (0.05M, pH 8,2 l) containing magnesium chloride (0.001M). The buffer was changed every 24 hours. The resultant solution was stored in a plastic tube at 4°C.

Coating the plastic beads

Polystyrene beads (2.2 mm) were thoroughly washed with distilled water. Each bead required 0.05 ml carbonate bicarbonate buffer pH 9.6. IgG was dissolved in buffer at a concentration of 100 µg ml⁻¹. The beads were added to the beaker containing the anti-rat IgG and buffer, sealed and incubated for 3 hours at 37°C, and then kept at 4°C until required.

Preparation of standard rat Immunoglobulin G

Rat IgG (10 mg) was resuspended in saline to give a final concentration of 5 mg ml⁻¹. Dilutions were carried out in veronal-buffered saline (VBS) containing Triton X 100 (0.01% v/v) prior to use.

Assay for rat Immunoglobulin G

Standard rat IgG dilutions were prepared together with dilution (1:4000) of serum from carrageenan-treated and control animals.

To plastic tubes, a single coated bead was added and washed (x3) in VBS-Triton X 100 buffer (500 µl). Samples of diluted sera were transferred to tubes (250 µl) and left at ambient temperature for 30 minutes. Excess protein was washed off (x3) with VBS-Triton X 100 (500 µl). Conjugate was prepared by dilution (1:100) with VBS-

Triton X 100 and added to each tube (250 μ l). Tubes were left at ambient temperature for 30 minutes and then washed (x3) with VBS-Triton X 100 (500 μ l).

Substrate was prepared by dissolving one 104 phosphatase substrate tablet in glycine-hydroxide buffer (5 ml) , then added to each tube (250 μ l) and left at ambient temperature for 6 minutes prior to the addition of sodium hydroxide solution (1.5M: 600 μ l) to terminate the reaction.

Absorbance of each sample was measured at 405 nm using an SP 1800 spectrophotometer and the protein concentration for each sample obtained from a calibration curve set up using pure IgG.

5.2 Haemagglutination assay

This assay was carried out using the method of Herbert (1967) to determine antibody formation in rat serum.

Rats were injected through the peritoneal cavity with 20 mg kg^{-1} polysaccharide, followed 24 hours later by 2.5×10^9 cells kg^{-1} , by the same route, of sheep red blood cells in saline.

The concentration of rat antibody for IgG is greatest at 8 days after antigen addition and so at this time the animals were bled by cardiac puncture and serum obtained.

The collected serum was complement-inactivated by immersion in a 56°C water bath for 30 minutes. Ovalbumin (0.1% w/v, 25 μ l) was added to wells (1-12) of microtitre plates and to well 1 serum (25 μ l) was added. The serum was serially diluted with a multichannel mixer.

Sheep red blood cells (1×10^9 cells ml^{-1} , 25 μ l) were added to each well and the well contents mixed and then incubated at 37°C

for 45 minutes. The titration end point was taken as the last well to show visible agglutination. Readings were taken as the inverse of the dilution factor, means obtained and the results expressed as the ratio of polysaccharide injected rat serum to saline rat serum.

6. LIPOPROTEIN STUDIES

6.1 Determination of serum cholesterol

Serum separation into its component lipoprotein fractions was carried out by a modified method of Hatch and Lees (1968).

Standard solutions with known densities were prepared with KBr at 20°C and measured by pycnometry. The following calculations were employed in the preparation of the solutions:

$$\text{Required density} = \frac{\text{Volume of fluid (required density - initial density)}}{\text{1-specific density of KBr (required density)}}$$

The solutions were prepared as seen in Table 6.1.1 and solutions of densities 1.045 g ml⁻¹ and 1.15 g ml⁻¹ were prepared using the above equation.

6.2 Lipoprotein separation

In an ultracentrifuge tube, serum (5 ml) was added to a 1.008 g ml⁻¹ solution (1.0 ml) and mixed. The tubes were carefully balanced (<0.001 g difference) and centrifuged at 108,000 x g for 16 hours at 14°C. The very low density lipoproteins (VLDL) were pipetted off, using the technique of deLalla, Elliott and Goffman (1954), to a marked 4 ml level. The VLDL was transferred to a 5 ml volumetric flask and made up to 5 ml with 1.008 g ml⁻¹ solution.

The remaining lipoprotein were thoroughly mixed with a solution of density 1.045 g ml⁻¹ (2 ml) and centrifuged at 108,000 x g for 16 hours at 14°C. The intermediate density lipoproteins (IDL) were removed as before and made up to 3 ml with 1.008 g ml⁻¹ solution.

To the centrifuge tube a 1.15 g ml⁻¹ solution (1 ml) was added

Table 6.1.1

Preparation of solutions for lipoprotein fractionation

Density 1.008 g ml⁻¹

Sodium chloride	11.40 g
Disodium EDTA	0.10 g
Sodium hydroxide (IM)	1.0 ml

A 1000 ml volumetric flask was filled to volume with distilled water followed by the addition of 3 ml of distilled water. Density was checked by pycnometry.

and the tubes centrifuged for 18 hours at $160,000 \times g$ at 14°C . The low density lipoproteins (LDL) were removed as before and made up to 5 ml with 1.008 g ml^{-1} solution. The remaining high density lipoproteins (HDL) were similarly made up to 5 ml volume.

All samples except VLDL were frozen at -20° until required. Standards were prepared from a stock solution of 40 mg ml^{-1} cholesterol.

6.3 Determination of serum fraction cholesterol

All samples and standards were assayed in duplicate. To each sample (200 μl) in a centrifuge tube was added ethanolic potassium hydroxide. The samples were thoroughly mixed and incubated at 37°C for 60 minutes. To each tube petroleum spirit was added (4 ml) followed by distilled water (2 ml). Mixing was carried out for 30 seconds per tube prior to centrifugation ($400 \times g$, 10 minutes, 20°C).

A volume of petroleum layer was removed, the amount depending on the fraction. For serum, control serum and LDL, 2 ml of petroleum was transferred to clean glass tubes and for VLDL, IDL and HDL 3 ml were similarly treated. All petroleum samples were dried under a stream of nitrogen.

Acid reagent comprising acetic acid: acetic anhydride: sulphuric acid was prepared in ratio 1.1:1.0:0.15 v/v. 6 ml of reagent was added to the tube and the tube contents mixed for 30 seconds before placing in a 25°C water bath for 30 minutes. Absorbance readings were obtained at 620 nm using an SP 1800 spectrophotometer and converted to ml per 100 ml^{-1} serum from the standard curve.

IV RESULTS

A. In vitro studies

A.1 Blood clotting assays

A.1.1 Whole blood clotting time

Polysaccharides, with the exception of K-carrageenan, exerted anticoagulant effects in the whole blood clotting assay (Graph A.1.1.1 p.78). Comparison of activity was determined on a weight basis with heparin showing anticoagulant activity (>20 min) at 0.005 mg ml^{-1} . Comparable anticoagulant activity was found with alkali-modified λ -, λ_{Na} - and λ_{Ca} -carrageenans at 0.5, 0.5 and 1.0 mg ml^{-1} respectively. A control value obtained from saline-treated plasma gave a clotting time of 4 minutes.

To examine the polysaccharide effects more closely the intrinsic pathway of coagulation was examined in a cell-free system.

A.1.2 Partial thromboplastin time clotting assay

Heparin was found to be anticoagulant ($>200 \text{ s}$) at 0.005 mg ml^{-1} , while concentrations of 0.01, 0.05 and 0.01 were required for alkali-modified λ -, λ_{Ca} - and λ_{Na} -carrageenan respectively to produce a similar response (Graph A.1.2.1, p.79). K-Carrageenan exhibited little anticoagulant activity even at high concentrations. A control value of $45.2 \pm 2.4 \text{ s}$ was obtained with saline-treated plasma.

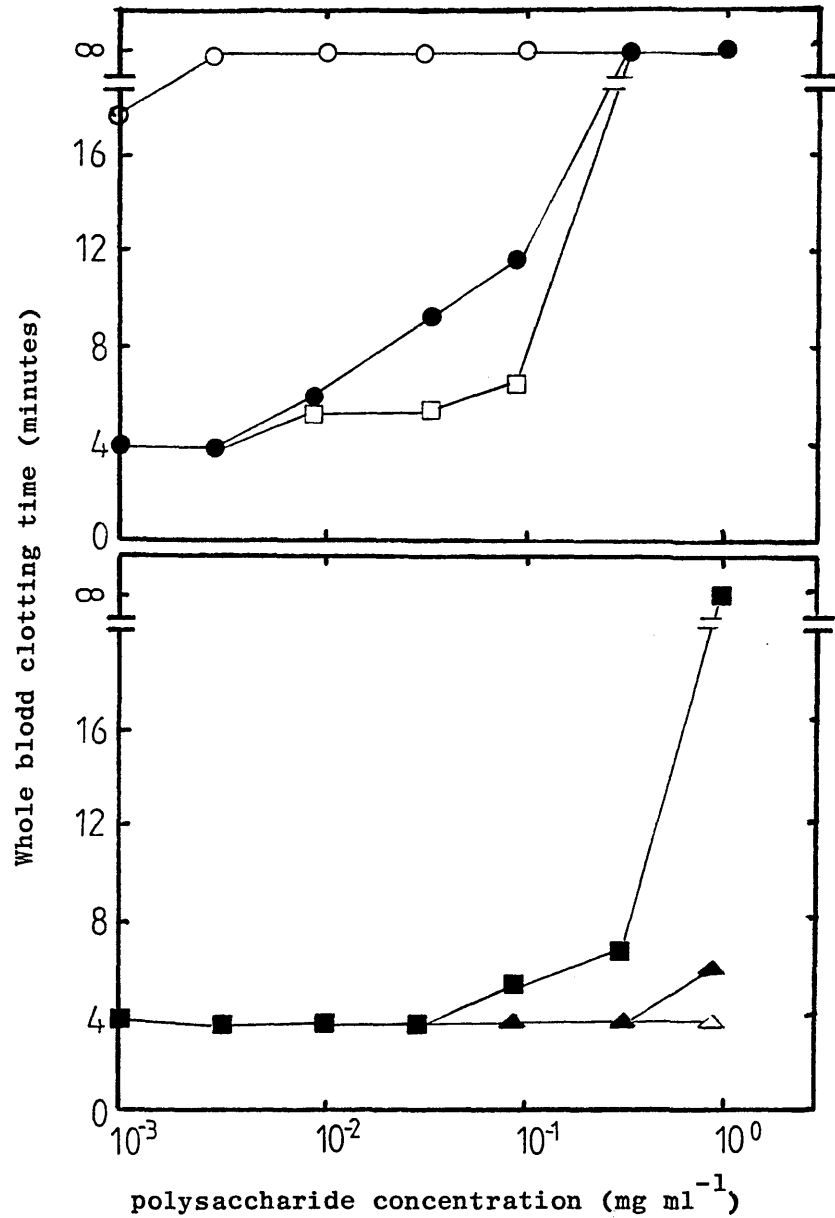
A.1.3 Partial thromboplastin time in Factor XII-depleted plasma

Results illustrated on Graph A.1.3.1, p.80 show that with a saline control

Graph A.1.1.1

The effect of heparin and carrageenan on whole blood

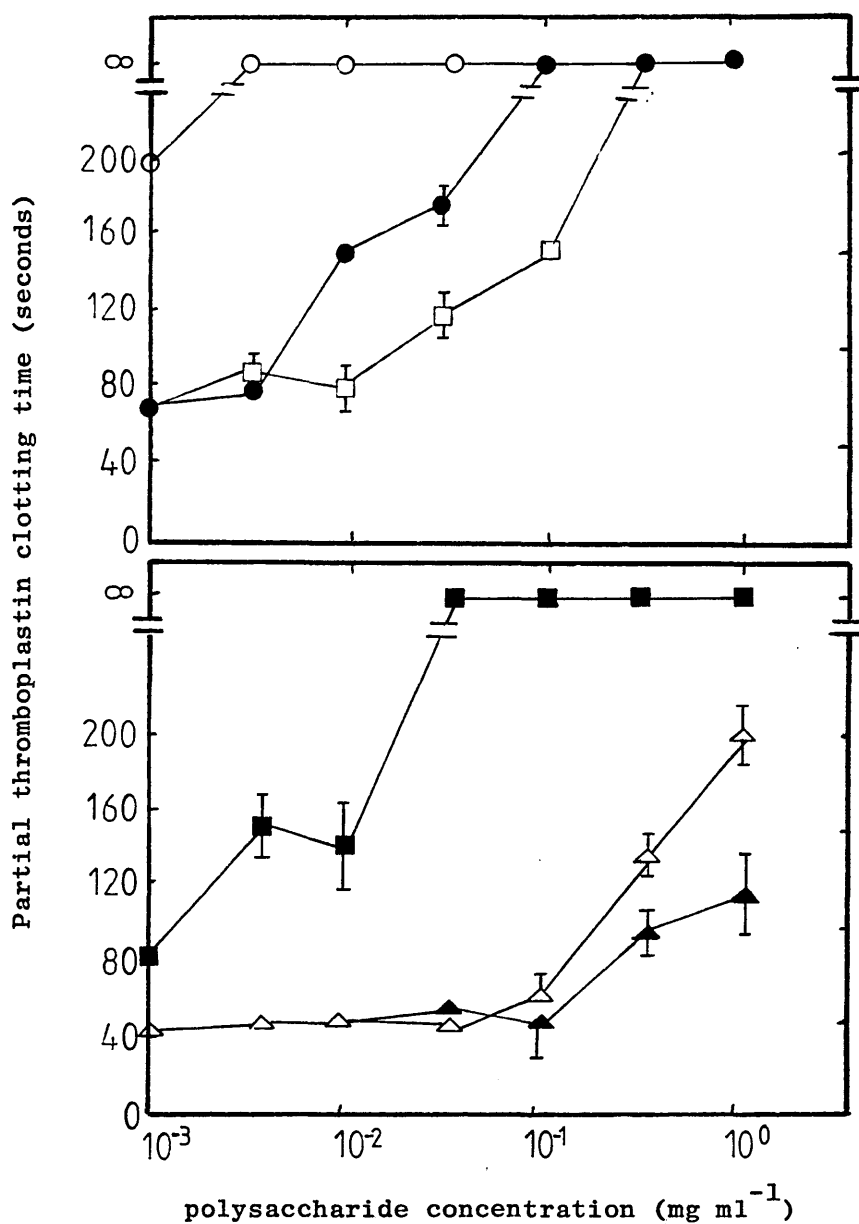
clotting time



The effect of heparin (O), alkali-modified λ -carrageenan (●), 1Na -carrageenan (□), 1Ca -carrageenan (■), 1Ca -carrageenan (▲) and 1K -carrageenan (▲) on whole blood clotting time. Control time was 4 minutes when $n=3$.

Graph A.1.2.1

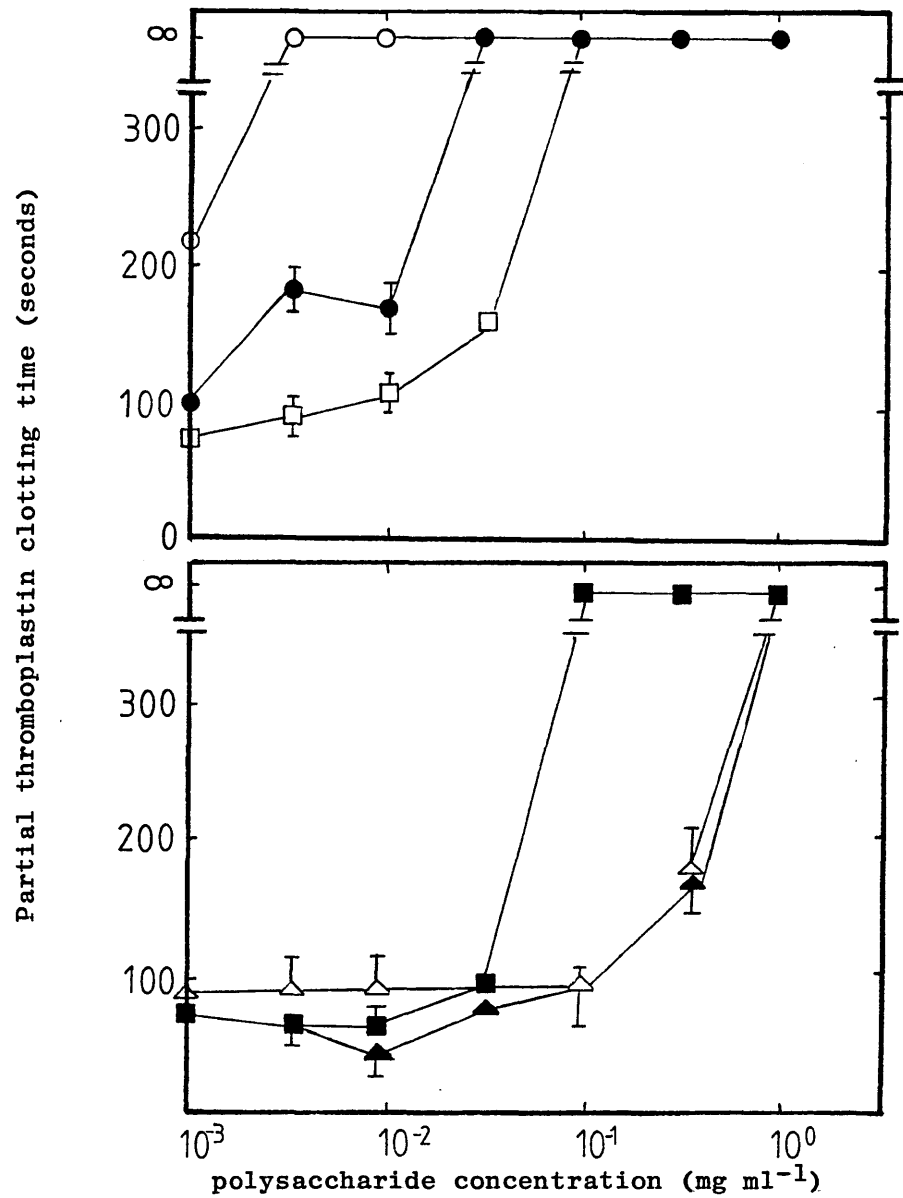
The effect of heparin and carrageenan on the partial
thromboplastin clotting time of plasma



Effect of heparin (O) alkali-modified λ -carrageenan (●), ι_{Na} -carrageenan (□) ι_{Ca} -carrageenan (◁), κ_{Ca} -carrageenan (◁) and κ_K -carrageenan (▲) on the PTT clotting time of plasma. Control time was 45.2 ± 2.4 s when $n=3$.

Graph A.1.3.1

The effect of heparin and carrageenan on the partial thromboplastin
clotting time of Factor XII depleted plasma



Effect of heparin (O), alkali-modified λ-carrageenan (●), ι_{Na}-carrageenan (□), ι_{Ca}-carrageenan (■), κ_{Ca}-carrageenan (△) and κ_K-carrageenan (▲) on the PTT clotting time Factor XII depleted plasma. Control time was 74.3 ± 0.7 s when n=3.

value of 74.3 ± 0.7 s , heparin and the carrageenans exhibited an anticoagulant effect (>400 s). Heparin and alkali-modified λ -carrageenan required concentrations of 0.005 and 0.05 mg ml⁻¹ respectively, while concentrations of 0.1 and 1.0 mg ml⁻¹ were required by the ι -carrageenans and κ -carrageenans to produce a similar response. The clotting time of Factor XII-depleted plasma in the presence of saline was 74.3 s .

A.1.4 Russell's viper venom clotting time

A control value of 15.3 ± 0.1 s was recorded for this assay. Anticoagulant activity (>100 s) was observed with all polysaccharides except the κ -carrageenans. Concentrations of 0.01 and 0.5 mg ml⁻¹ were required by heparin and ι_{Ca} -carrageenan respectively to produce an anticoagulant effect while 1.0 mg ml⁻¹ was required by alkali-modified λ -carrageenan to produce a similar response (Graph A.1.4.1, p.82).

A.1.5 Russell's viper venom clotting time in Factor XII depleted plasma

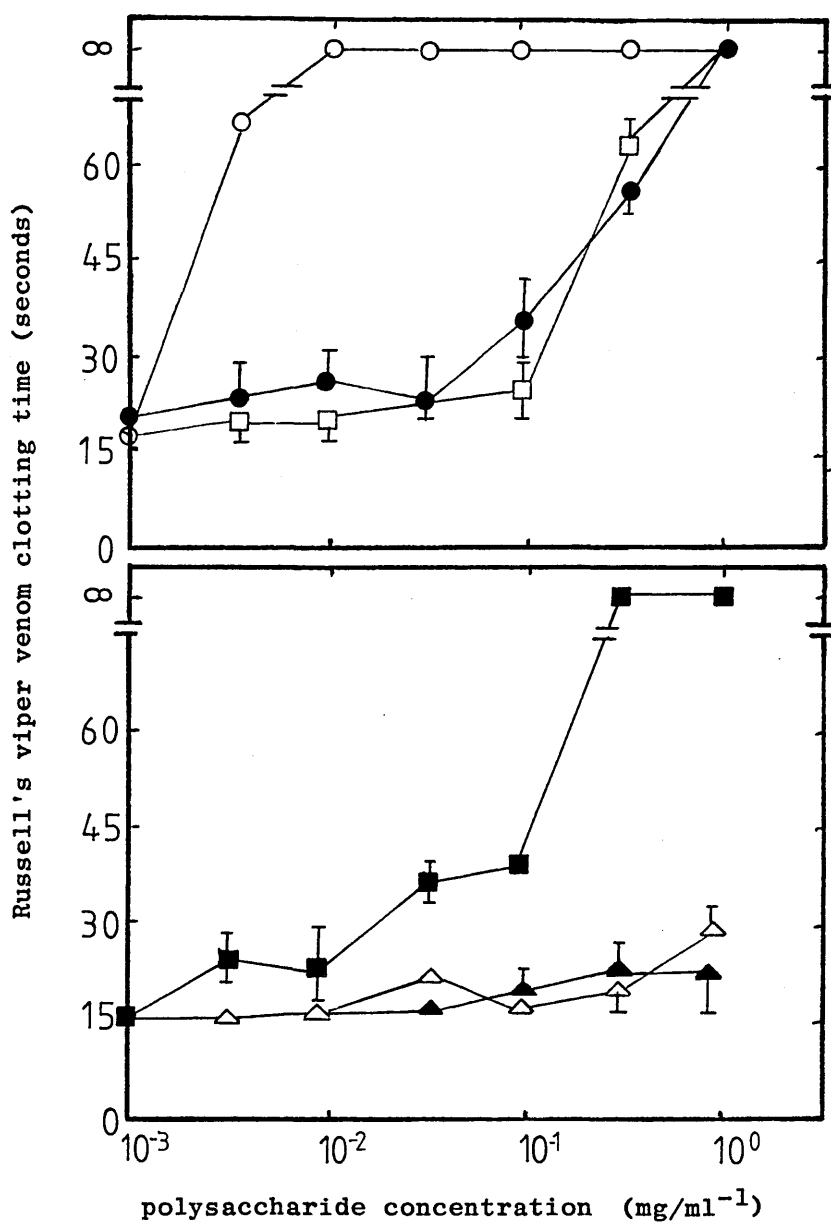
Results obtained for this assay were similar to those recorded for normal plasma with a slight shift in concentration requirements for heparin from 0.01 mg ml⁻¹ to 0.05 mg ml⁻¹. The alkali-modified λ - and ι_{Ca} -carrageenan were found to be anticoagulant (>100 s) at concentrations of 0.5 mg ml⁻¹ while ι_{Na} -carrageenan required 1.0 mg ml⁻¹ for a similar response (Graph A.1.5.1, p.83). κ -Carrageenans exhibited no effect. Control values were found to be 14.5 ± 0.4 s .

A.1.6 Russell's viper venom clotting time in antithrombin III-depleted plasma

In AT III-depleted plasma, heparin anticoagulant activity was greatly reduced, even at high concentrations. With a control value of

Graph A.1.4.1

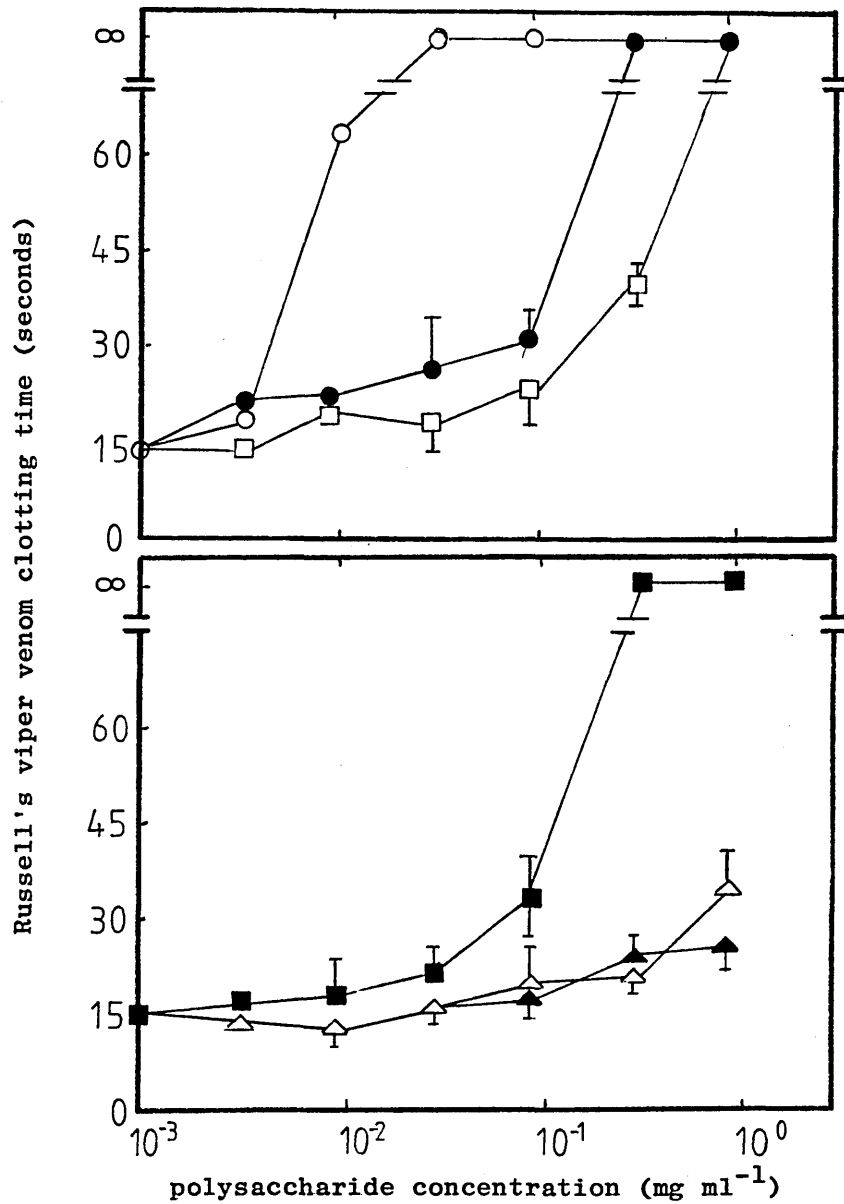
The effect of heparin and carrageenan on Russell's
viper venom clotting time of plasma



Effect of heparin (O), alkali-modified λ -carrageenan (●), λ_{Na} -carrageenan (□), λ_{Ca} -carrageenan (■), κ_{Ca} -carrageenan (△) and κ_K -carrageenan (▲) on RVV clotting time of plasma. Control time was 15.3 ± 0.1 s when $n=3$.

Graph A.1.5.1

The effect of heparin and carrageenan on Russell's
viper venom clotting time on Factor XII
depleted plasma



Effect of heparin (O), alkali-modified λ -carrageenan (●), λ -Na-carrageenan (□), λ -Ca-carrageenan (■), κ -Ca-carrageenan (△) and κ -K-carrageenan (▲) on the RVV clotting time on Factor XII depleted plasma. Control time was 14.5 ± 0.4 s when $n=3$.

37.4 ± 2.6 l_{Na}^- , l_{Ca}^- - and alkali-modified λ -carrageenan were found to be anticoagulant (>100 s) at 0.1, 0.5 and 1.0 mg ml^{-1} respectively. As before, treatment of plasma with κ -carrageenan did not affect the coagulant activity (Graph A.1.6.1, p.85).

A.1.7 Thrombin clotting time assay

All polysaccharides exerted an anticoagulant effect (>100 s). The concentrations required to obtain an anticoagulant effect for heparin and l_{Na}^- -carrageenan were 0.005 and 0.01 mg ml^{-1} respectively. Both alkali-modified λ - and l_{Ca}^- -carrageenan required a concentration of 0.05 mg ml^{-1} , while the κ -carrageenans showed an anticoagulant effect at 0.5 mg ml^{-1} (Graph A.1.7.1, p.86). Control values were 22.2 ± 1.0 s .

A.1.8 Thrombin clotting time in antithrombin III-depleted plasma

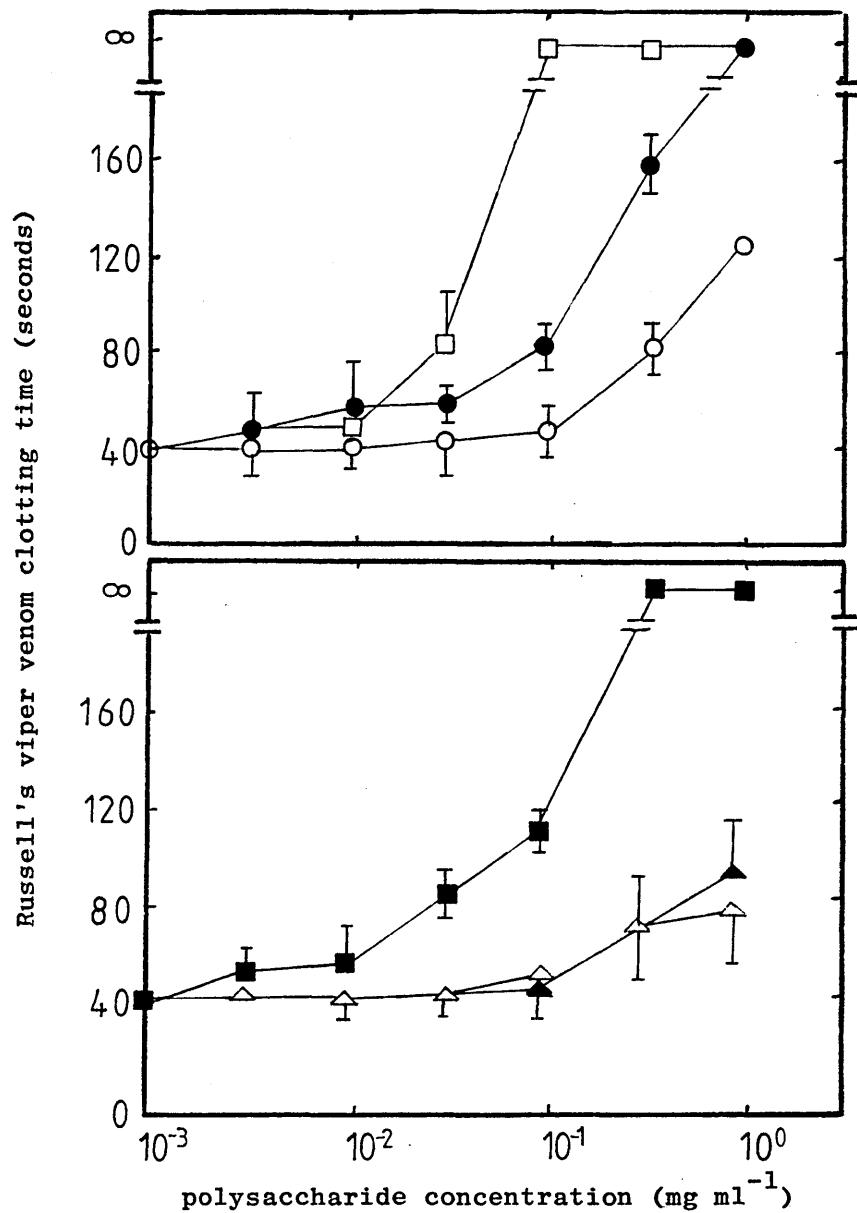
Heparin anticoagulant activity (>200 s) was completely suppressed in the absence of AT III while, in the absence of AT III, all carrageenans remained anticoagulant at concentrations of 0.5 mg ml^{-1} or above (Graph A.1.8.1, p.87). With control values of $50.0 \text{ s} \pm 4.5$ in this test system, procoagulant effects were observed with alkali-modified λ -, l_{Na}^- and κ_{K} -carrageenan at 0.001 mg ml^{-1} and with κ_{Ca}^- and l_{Ca}^- -carrageenan at 0.05 mg ml^{-1} and below.

A.1.9 Factor X (Factor Xa)-catalysed amidolytic assay

Results expressed in Graph A.1.9.1, p. 88 illustrate that only heparin inhibited the amidolytic activity of Factor X (Factor Xa), an effect which occurred maximally between 0.05 - 0.1 mg ml^{-1} . Slight inhibition was observed with κ_{K} -carrageenan at 0.05 mg ml^{-1} but all

Graph A.1.6.1

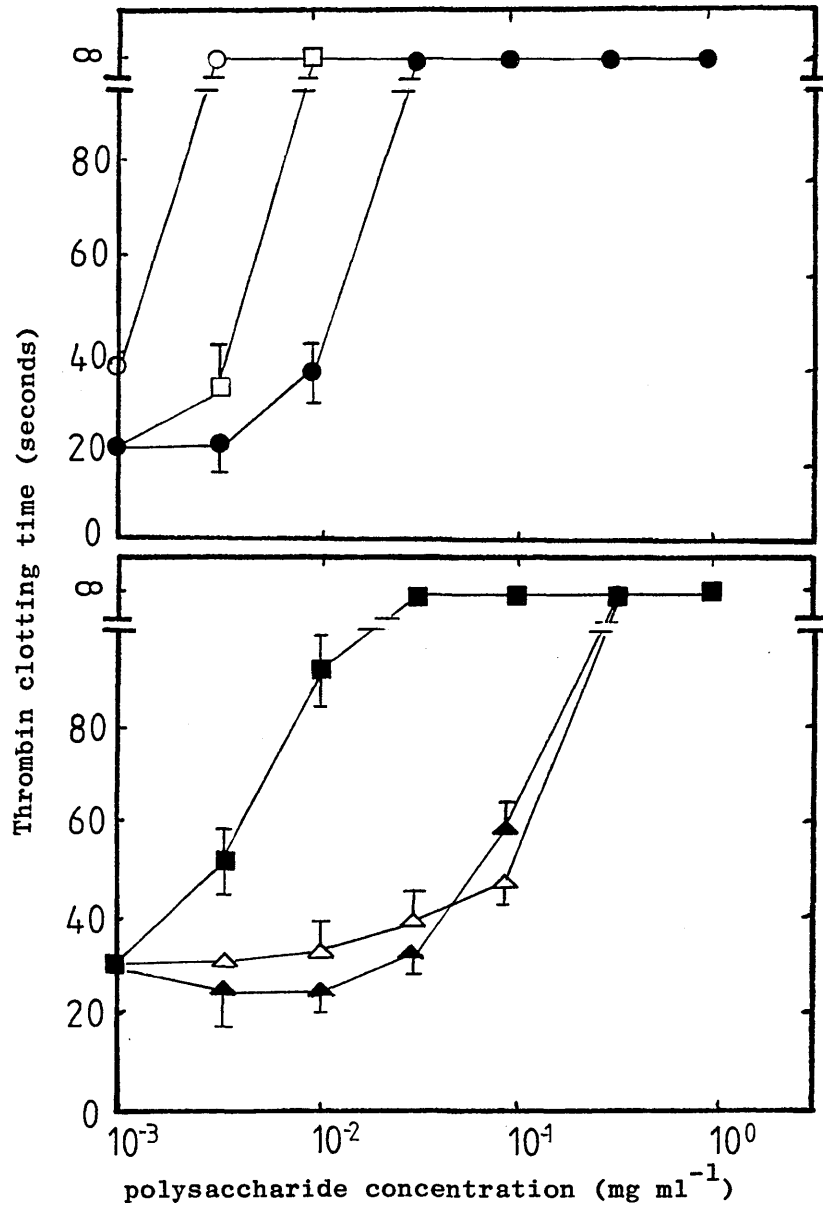
The effect of heparin and carrageenan on Russell's
viper venom clotting time in plasma
depleted of antithrombin III



Effect of heparin (O), alkali-modified λ -carrageenan (●), ι_{Na} -carrageenan (□), ι_{Ca} -carrageenan (■), κ_{Ca} -carrageenan (△) and κ_K -carrageenan (▲) on RVV clotting time of AT III depleted plasma. Control time was 37.4 ± 2.6 s when $n=3$.

Graph A.1.7.1

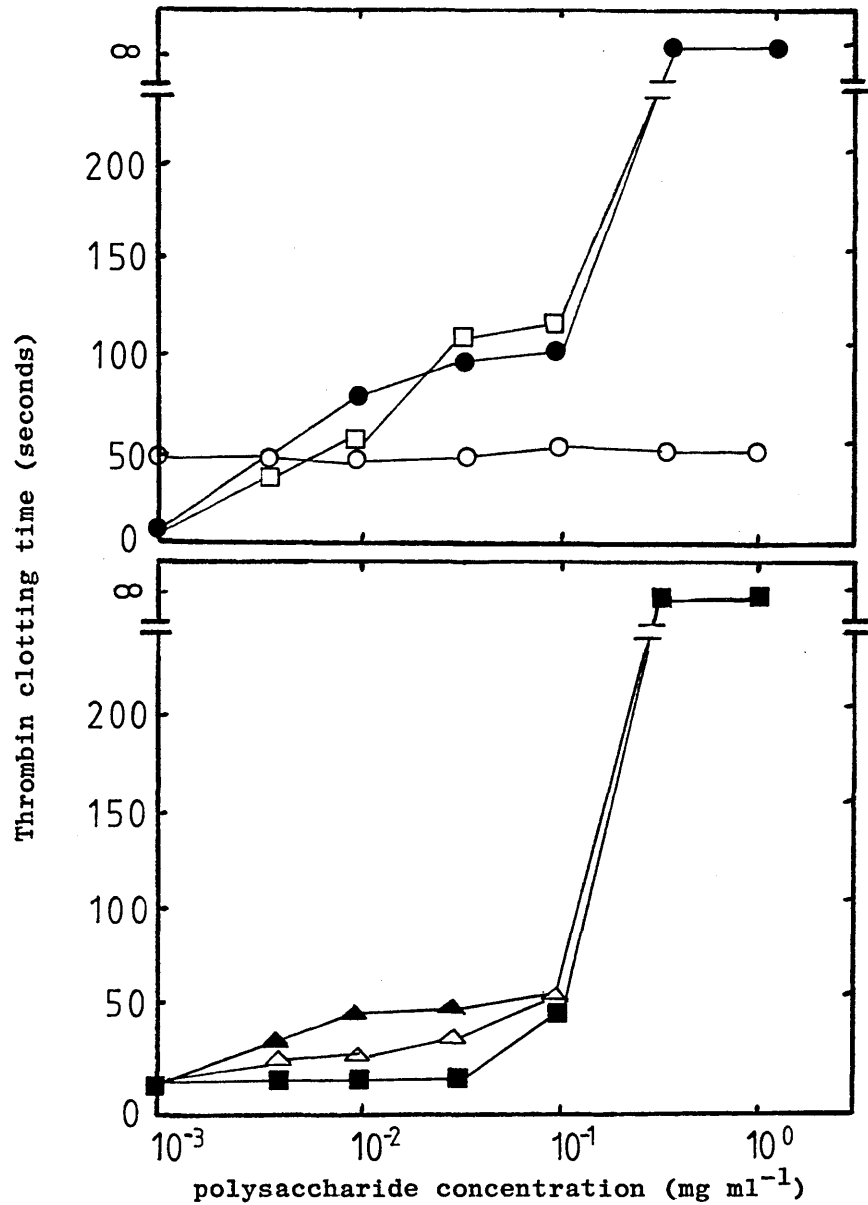
The effect of heparin and carrageenan on
the thrombin clotting time of plasma



The effect of heparin (O), alkali-modified λ -carrageenan (●), 1_{Na} -carrageenan (□), 1_{Ca} -carrageenan (■), K_{Ca} -carrageenan (△) and K_K -carrageenan (▲) on the thrombin clotting of plasma. Control time was 22.2 ± 1.0 s when $n=3$.

Graph A.1.8.1

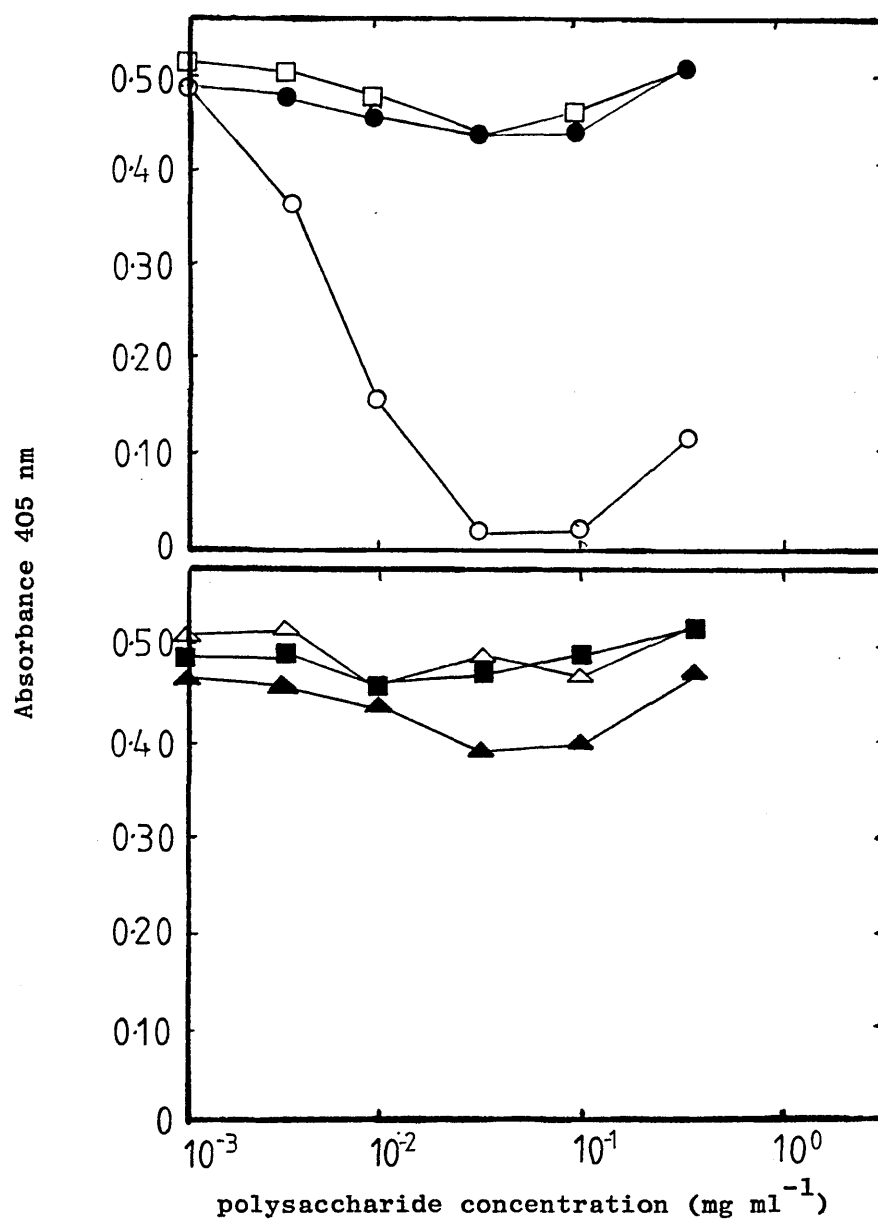
The effect of heparin and carrageenan on the
thrombin clotting time in antithrombin III
depleted plasma



Effect of heparin (O), alkali-modified λ -carrageenan (●), 1_{Na} -carrageenan (□), 1_{Ca} -carrageenan (■), κ_{Ca} -carrageenan (△) and κ_{K} -carrageenan (▲) on the thrombin clotting time of plasma depleted of AT III. Control time was 50.0 ± 4.5 s when $n=3$.

Graph A.1.9.1

The effect of heparin and carrageenan on Factor X
(Factor Xa) amidolytic activity



Effect of heparin (O), alkali-modified λ-carrageenan (●), ι_{Na}-carrageenan (□), ι_{Ca}-carrageenan (■), κ_{Ca}-carrageenan (△) and κ_K-carrageenan (▲) on Factor X (Factor Xa) activation. Control value of 0.5 ± 0.02 nm when n=3.

other carrageenans had no effect.

A.1.10 Thrombin-catalysed amidolytic assay

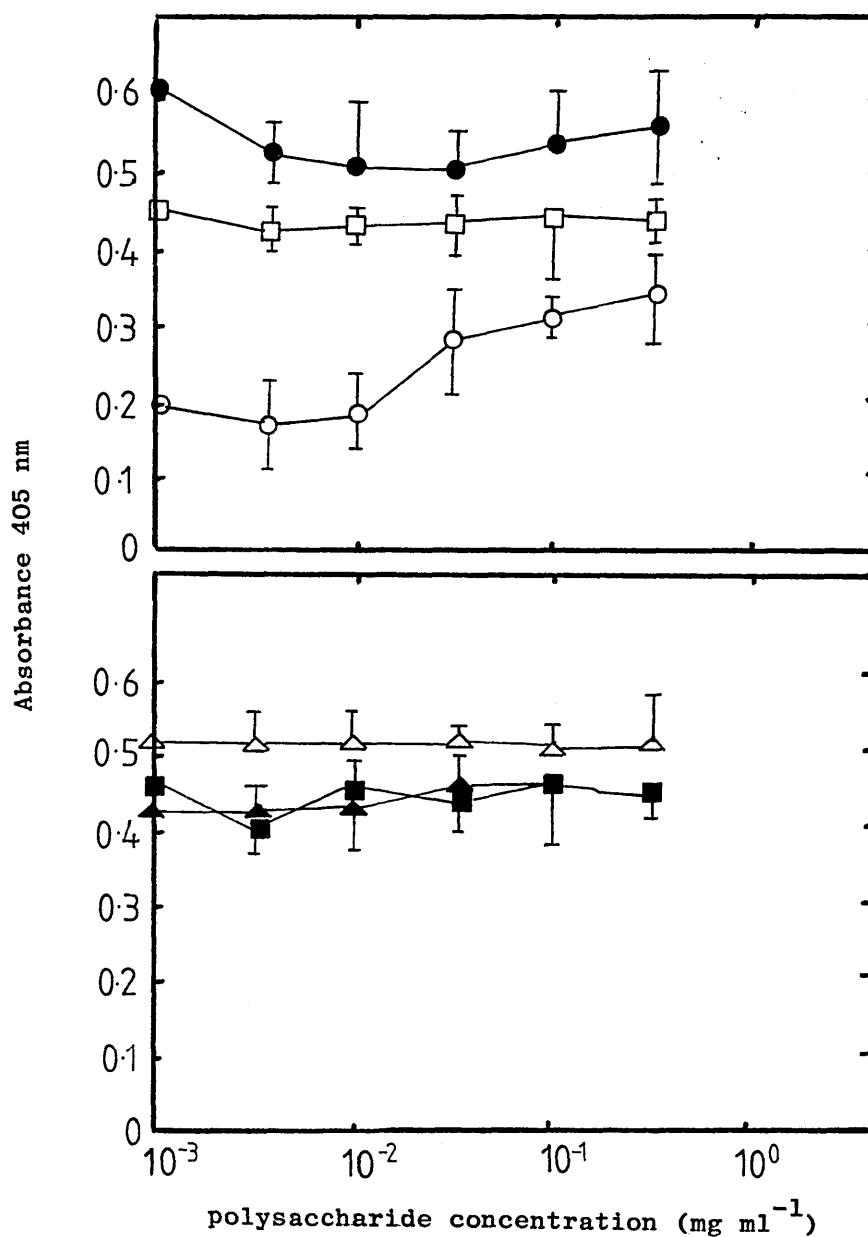
Heparin, over the range of concentrations tested, reduced thrombin amidolytic activity below the control value of $OD = 0.52 \pm 0.01$ (Graph A.1.10.1, p.90). The carrageenans had little or no effect on this system when measured via the end point assay.

In an attempt to elucidate the carrageenan effects on thrombin the experiment was repeated but, on addition of chromogenic substrate S2251, a recorder, attached to the spectrophotometer, was started. The rate of paranitroaniline (pNA) released was recorded for each polysaccharide concentration and tracings superimposed for ease of comparison (Graph A.1.10.2 - A.1.10.4, p.91-93).

Heparin (Graph A.1.10.2, p.91) inhibited thrombin-catalysed amidolysis at concentrations above 0.01 mg ml^{-1} alkali-modified λ - and κ -carrageenan (Graphs A.1.10.3 - A.1.10.4, p.92 and 93) both showed inhibition of thrombin-catalysed amidolysis at 1.0 mg ml^{-1} only. No effects were observed with the other carrageenans.

Graph A.1.10.1

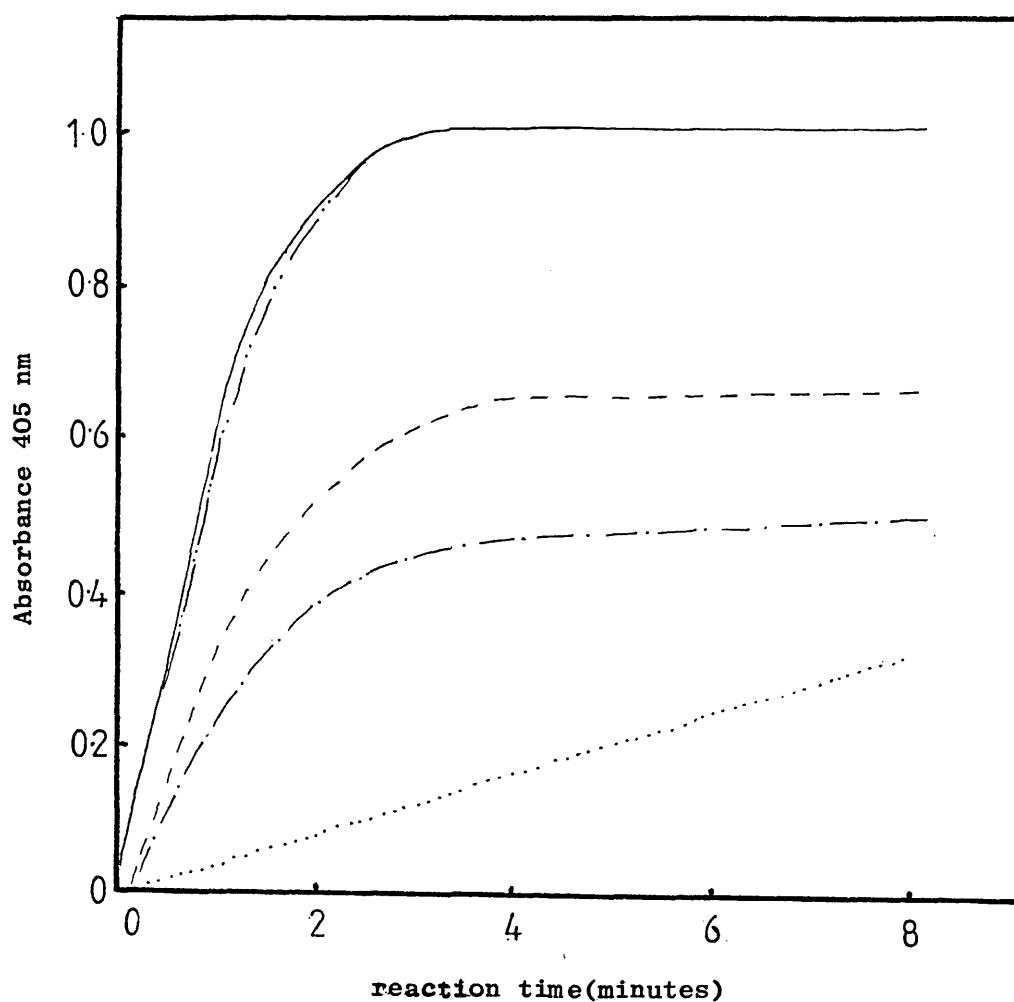
The effect of heparin and carrageenan on thrombin-catalysed
amidolytic activity in plasma



Effect of heparin (O), alkali-modified λ-carrageenan (●), ι_{Na}-carrageenan (□), ι_{Ca}-carrageenan (■), κ_{Ca}-carrageenan (△) and κ_K-carrageenan (▲) on thrombin activity in plasma. Control value of 0.52 ± 0.01 nm when n=3.

Graph A.1.10.2

The effect of heparin on thrombin-catalysed amidolytic activity
determined by the measurement of the rate of reaction

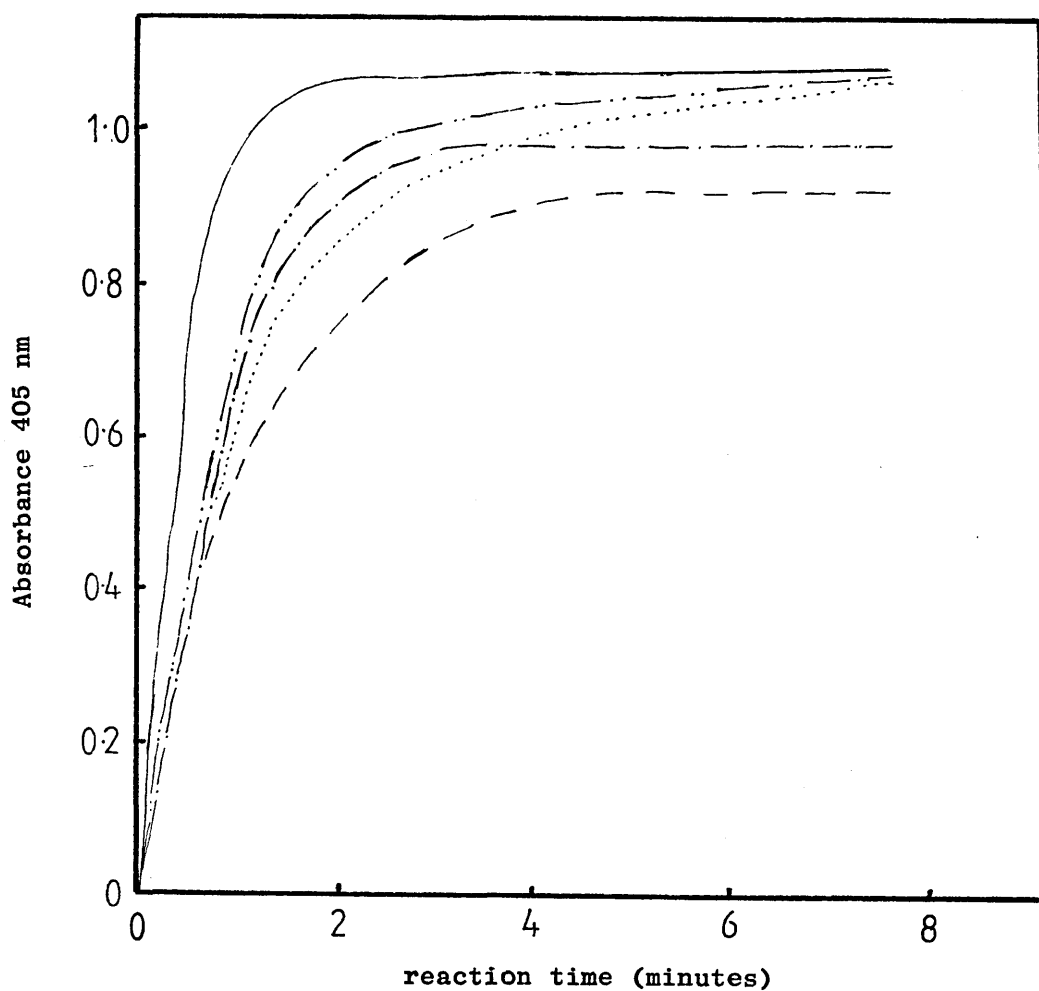


Heparin was preincubated with plasma prior to addition of thrombin. The initial rate of reaction was measured when substrate was added at zero time. Heparin was present in the following concentrations:

- no heparin
- - - 1.0 mg ml⁻¹
- . - 0.1 mg ml⁻¹
- 0.01 mg ml⁻¹
- . - . 0.001 mg ml⁻¹

Graph A.1.10.3

The effect of alkali-modified λ -carrageenan on thrombin-catalysed
amidolytic activity determined by the measurement of the
rate of reaction

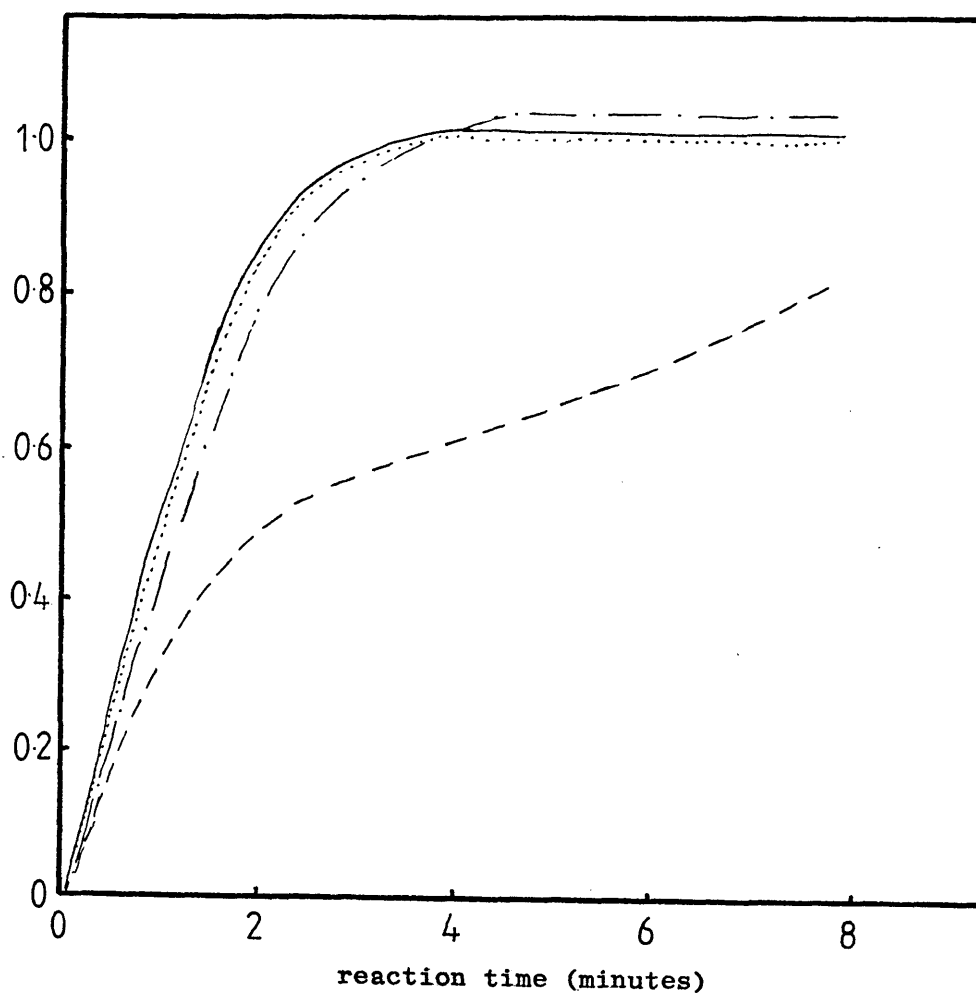


Alkali-modified λ -carrageenan was preincubated with plasma prior to the addition of thrombin then substrate was added at zero time. Alkali-modified λ -carrageenan was present in the following final concentrations:

— no carrageenan
----- 1.0 mg ml⁻¹
-.-.- 0.1 mg ml⁻¹
..... 0.01 mg ml⁻¹
-.-.-. 0.001 mg ml⁻¹

Graph A.1.10.4

The effect of the ^1Na -carrageenan on thrombin-catalysed
amidolytic activity determined by the measurement
of the rate of reaction



^1Na -Carrageenan was preincubated with plasma prior to addition of thrombin then the substrate was added at zero time. ^1Na -Carrageenan was present in the following concentrations:

— no carrageenan
--- 1.0 mg ml⁻¹
-.- 0.1 mg ml⁻¹
..... 0.01 mg ml⁻¹
- - - - 0.001 mg ml⁻¹

A.2 Fibrinolysis

A.2.1 Euglobulin-derived fibrinolytic activity

Results (Graph A.2.1.1, p.95) show that heparin had no effect on fibrinolysis (control of $32.8 \pm 4.1 \text{ mm}^2$). The carrageenans potentiated euglobulin-derived fibrinolytic activity at a concentration of 0.1 mg ml^{-1} . Of the carrageenan fractions tested, κ -carrageenan was observed to produce the greatest response with a four-fold increase in activity over the control value. The alkali-modified λ - and κ -carrageenan fractions increased the euglobulin fibrinolytic response with a 2.0 - 2.5 fold increase in fibrinolysis.

A.2.2 Inhibitor studies with ϵ -aminocaproic acid

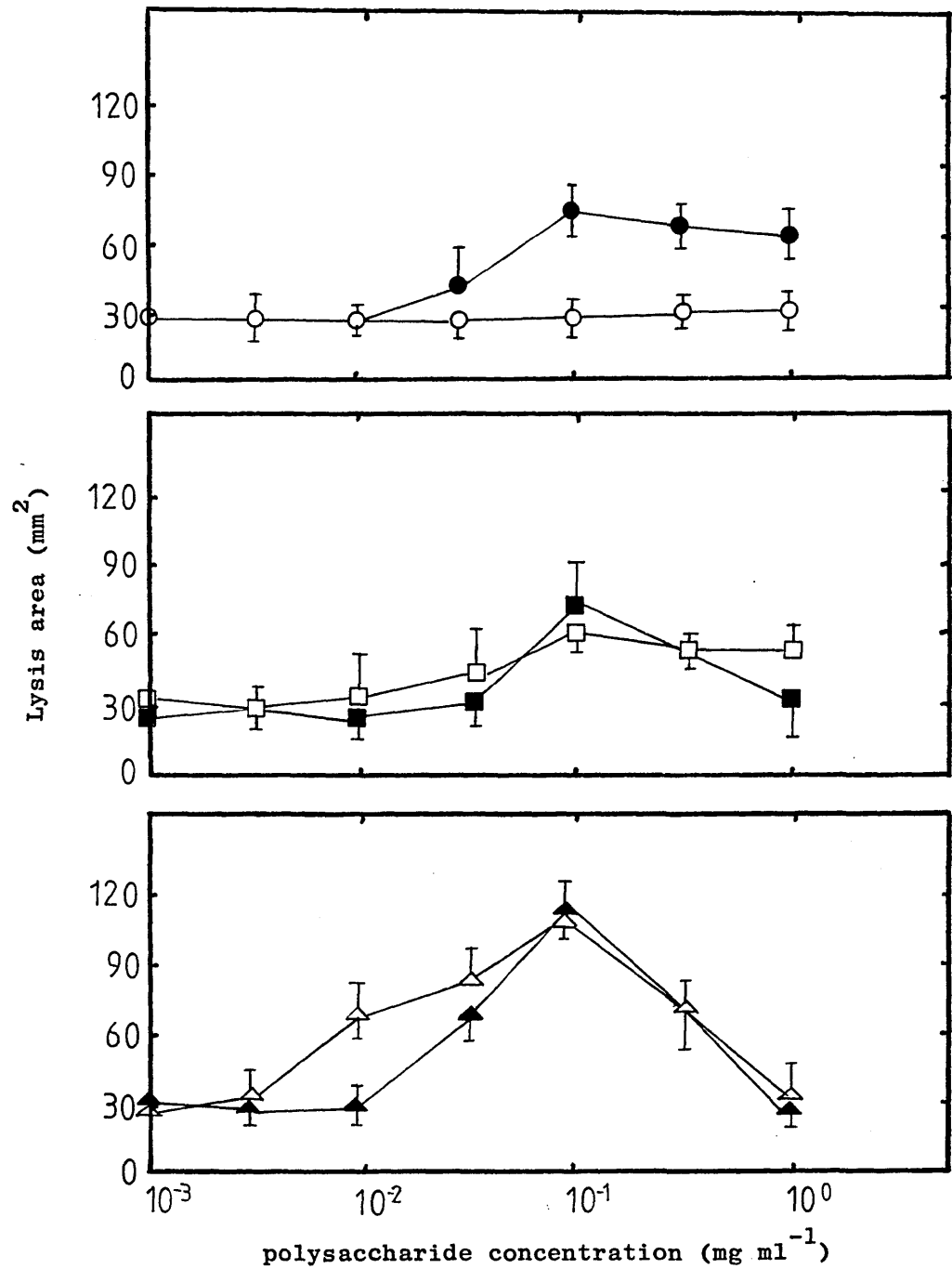
The incorporation of ϵ -ACA (2 mM) into the plasma before euglobulin precipitation resulted in the inhibition of the alkali-modified λ - and λ -carrageenan fibrinolytic response (Graph A.2.2.1, p. 96). Heparin, as before, exhibited no effect. The κ -carrageenans maintained their fibrinolytic activity, which was maximal at 0.1 mg ml^{-1} , with the κ_K -carrageenan showing greater activity than the κ_{Ca} -carrageenan. Control values were in the range $23.4 \pm 1.25 \text{ mm}^2$.

A.2.3 Inhibitor studies with flufenamic acid

Flufenamic acid (12 mM), incorporated into the plasma prior to euglobulin precipitation, resulted in fibrinolytic activity with alkali-modified λ -, λ_{Ca} -, κ_K -and κ_{Ca} -carrageenan at 0.10 mg ml^{-1} (Graph A.2.3.1, p.97), although no potentiation over the fibrinolytic response observed in normal plasma was seen (Graph A.2.1.1, p.95). Heparin had no activity in this system. Control values were found to be $49.3 \pm 1.0 \text{ mm}^2$.

Graph A.2.1.1

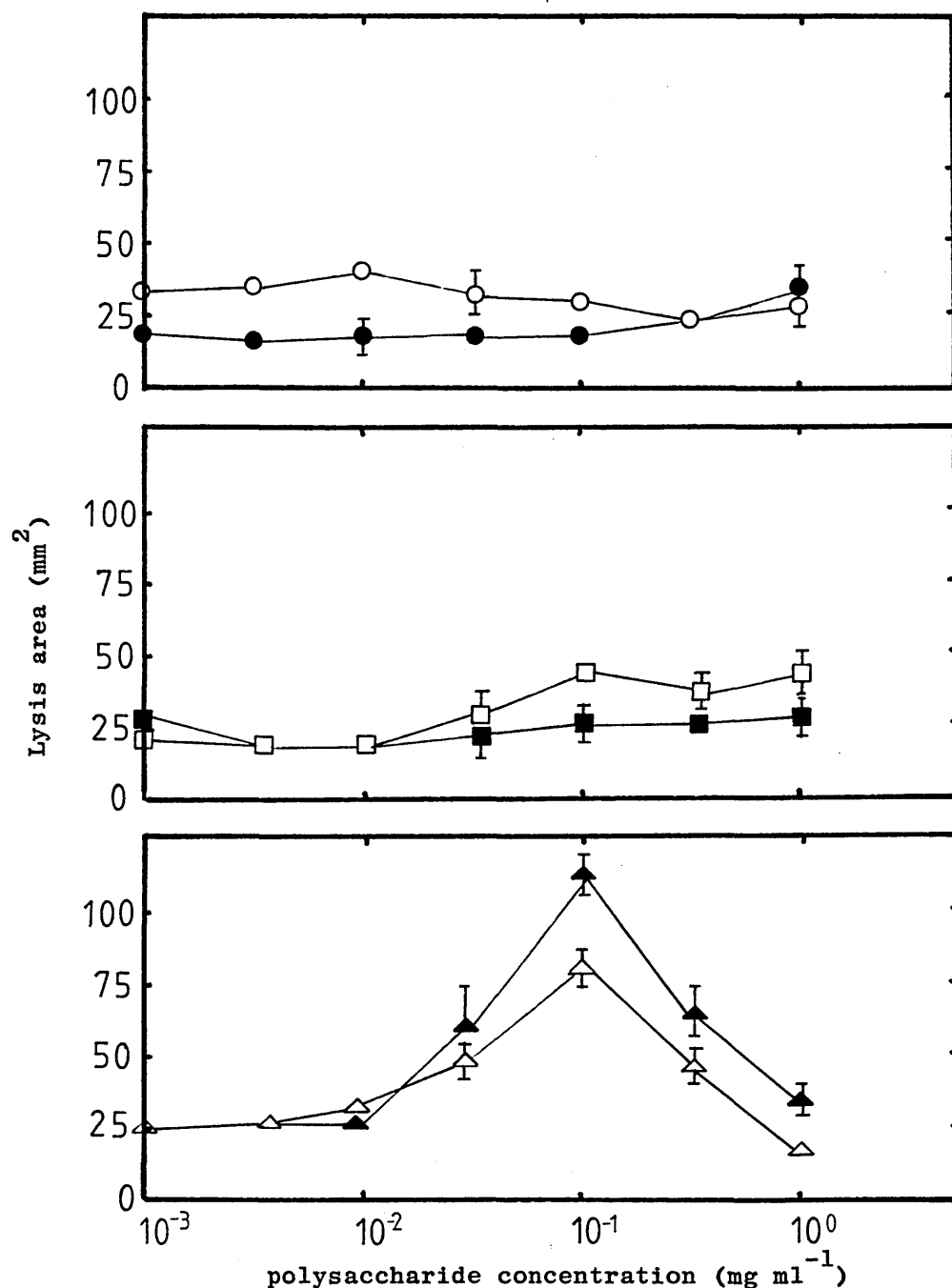
The effect of heparin and carrageenan on
euglobulin fibrinolytic activity



Effect of heparin (O), alkali-modified λ-carrageenan (●), 1_{Na}-carrageenan (□), 1_{Ca}-carrageenan (■), K_{Ca}-carrageenan (△) and K_K-carrageenan (▲) on euglobulin fibrinolytic activity. Control area of 32.8 ± 0.1 mm² when n=2.

Graph A.2.2.1

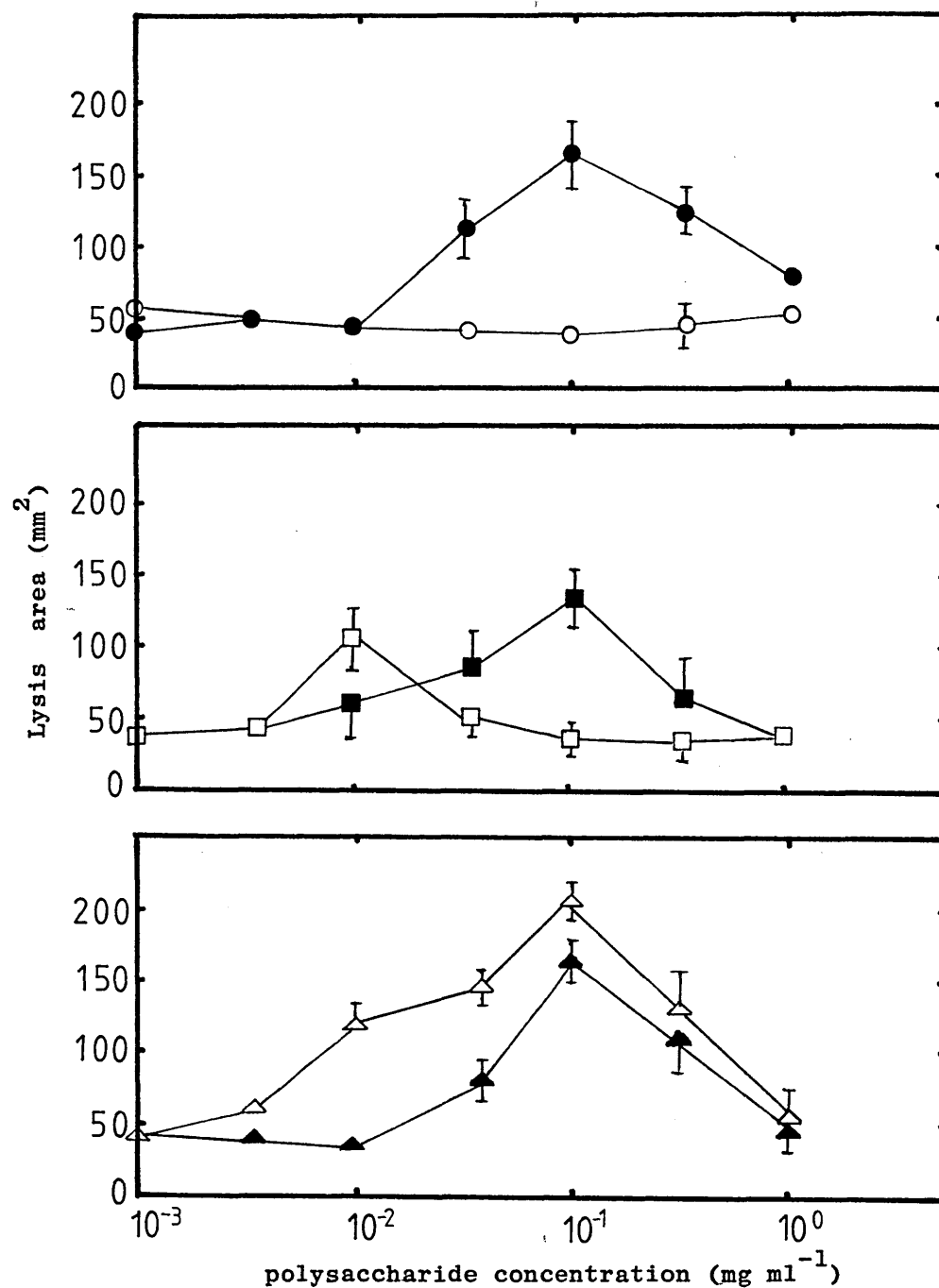
The effect of ϵ -ACA on heparin and carrageenan
derived euglobulin fibrinolytic activity



Effect of heparin (○), alkali-modified λ -carrageenan (●), ^1Na -carrageenan (□), ^1Ca -carrageenan (■), ^1Ca -carrageenan (△) and ^1K -carrageenan (▲) on plasma with ϵ -ACA incorporated prior to euglobulin precipitation. Control area was $23.4 \pm 1.25 \text{ mm}^2$ when $n=2$.

Graph A.2.3.1

The effect of flufenamic acid on heparin and carrageenan
derived euglobulin fibrinolytic activity



Effect of heparin (O), alkali-modified λ-carrageenan (●), ι_{Na} -carrageenan (□), ι_{Ca} -carrageenan (■), κ_{Ca} -carrageenan (△) and κ_K -carrageenan (▲) on plasma with flufenamic acid incorporated prior to euglobulin precipitation. Control area was 49.3 ± 1.0 mm² when n=2.

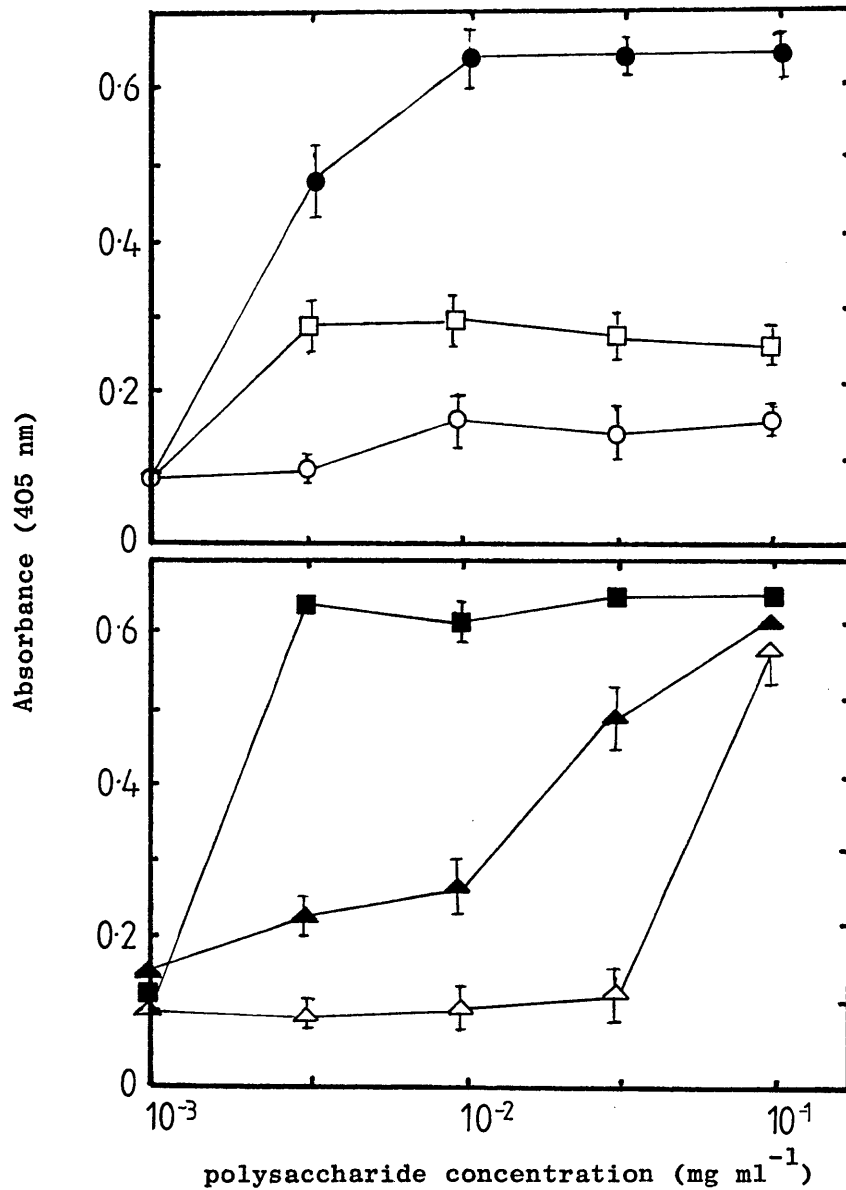
A.3 The kallikrein-kinin system

A.3.1 Amidolytic assay for carrageenan derived kallikrein activation

The release of paranitroaniline, as measured by spectrophotometry, indicates the activation of prekallikrein by the carrageenans. Only alkali-modified λ - and κ -carrageenan fractions increased amidolysis at concentrations above 0.005 mg ml^{-1} . Little or no activity was observed with heparin or other carrageenan samples (Graph A.3.1.1 p. 99). Control values were in the range 0.1 ± 0.002 at A_{405} .

Graph A.3.1.1

The effect of heparin and carrageenan on the prekallikrein
as shown by amidolysis



Effect of heparin (○), alkali-modified λ-carrageenan (●), λ_{Na}-carrageenan (□), λ_{Ca}-carrageenan (■), κ_{Ca}-carrageenan (△) and κ_K-carrageenan (▲) on prekallikrein amidolytic activity. Control absorption was 0.1 ± 0.002 at A₄₀₅ when n=2.

A.4 Blood platelet studies

A.4.1 Effect of carrageenan on platelet aggregation

In all experiments heparin and carrageenan failed to induce aggregation of platelets when tested in platelet-rich plasma. Platelet samples were viewed by light microscopy and only at high polysaccharide concentrations (1.0 mg ml^{-1}) were clusters of 4-10 platelets observed. These platelets showed a normal discoid shape with no pseudopodia evident, Introduction p. 30.

A.4.2 Effect of carrageenan on adenosine 5'-diphosphate-induced aggregation

No change in the aggregation response to $10 \text{ } \mu\text{M}$ ADP was observed in the presence of polysaccharide (range $1.0 - 0.001 \text{ mg ml}^{-1}$). Platelets viewed microscopically were seen to have a normal discoid shape with clusters of 4-12 platelets found only at high (1.0 mg ml^{-1}) polysaccharide concentration.

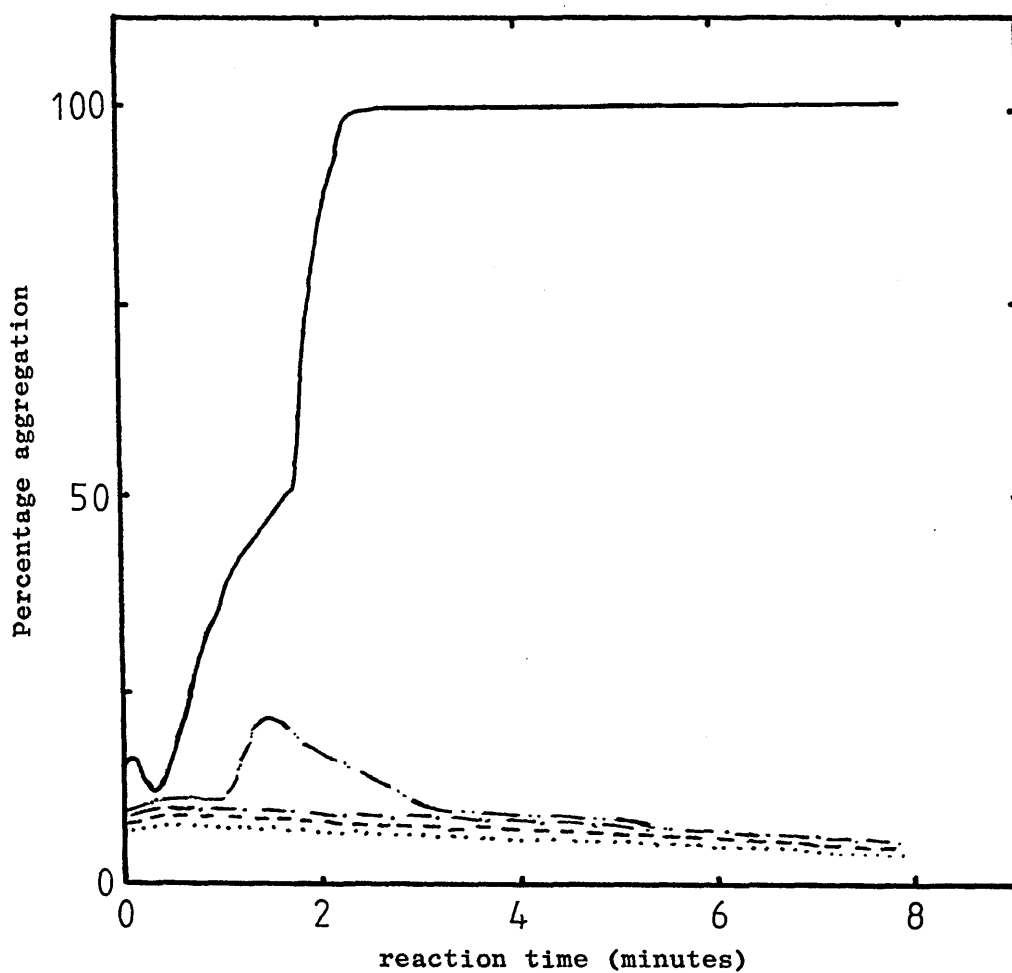
A.4.3 Effect of carrageenan on thrombin-induced aggregation

A concentration of $1.0 \text{ NIH U ml}^{-1}$ of thrombin induced a distinct biphasic response. The aggregation tracings obtained for each polysaccharide were superimposed for ease of comparison.

Heparin at all concentrations tested ($1.0 - 0.001 \text{ mg ml}^{-1}$), inhibited thrombin-induced aggregation, as shown by the absence of thrombus formation (Graph A.4.3.1, p.101). Alkali-modified λ -carrageenan inhibited thrombin at concentrations of 0.01 mg ml^{-1} and above (Graph A.4.3.2, p.102) while both ι -carrageenan salts inhibited at 1.0 and 0.1 mg ml^{-1} (Graphs A.4.3.3 and A.4.3.4, p. 103, 104). Both κ -carrageenans inhibited thrombin-induced aggregation at 1.0 mg ml^{-1} only (Graphs A.4.3.5 and A.4.3.6, p. 105, 106).

Graph A.4.3.1

The effect of heparin on thrombin-induced
platelet aggregation

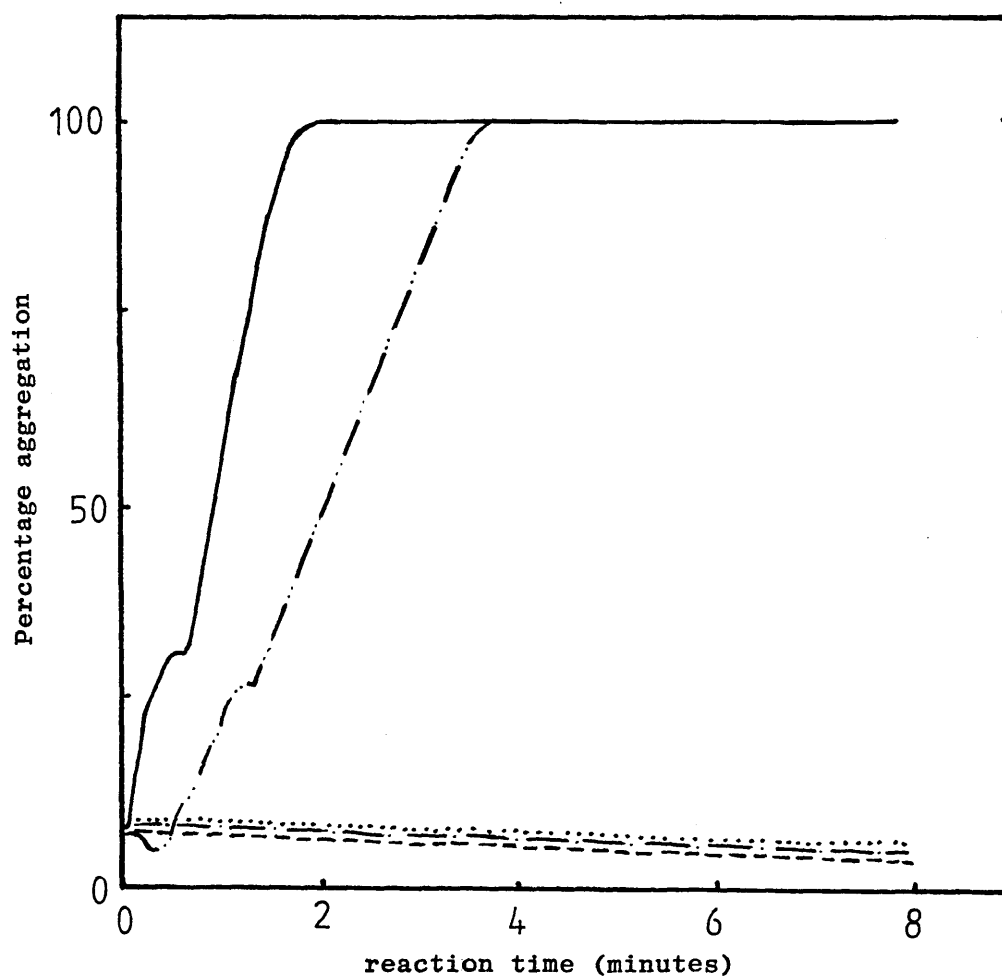


Heparin was incubated with PRP for 3 minutes followed by thrombin (1.0 NIH U ml⁻¹) addition at zero time.

— control
---- heparin (1.0 mg ml⁻¹)
- . - . heparin (0.1 mg ml⁻¹)
..... heparin (0.01 mg ml⁻¹)
- . . . heparin (0.001 mg ml⁻¹)

Graph A.4.3.2

The effect of alkali-modified λ -carrageenan on
thrombin-induced platelet aggregation

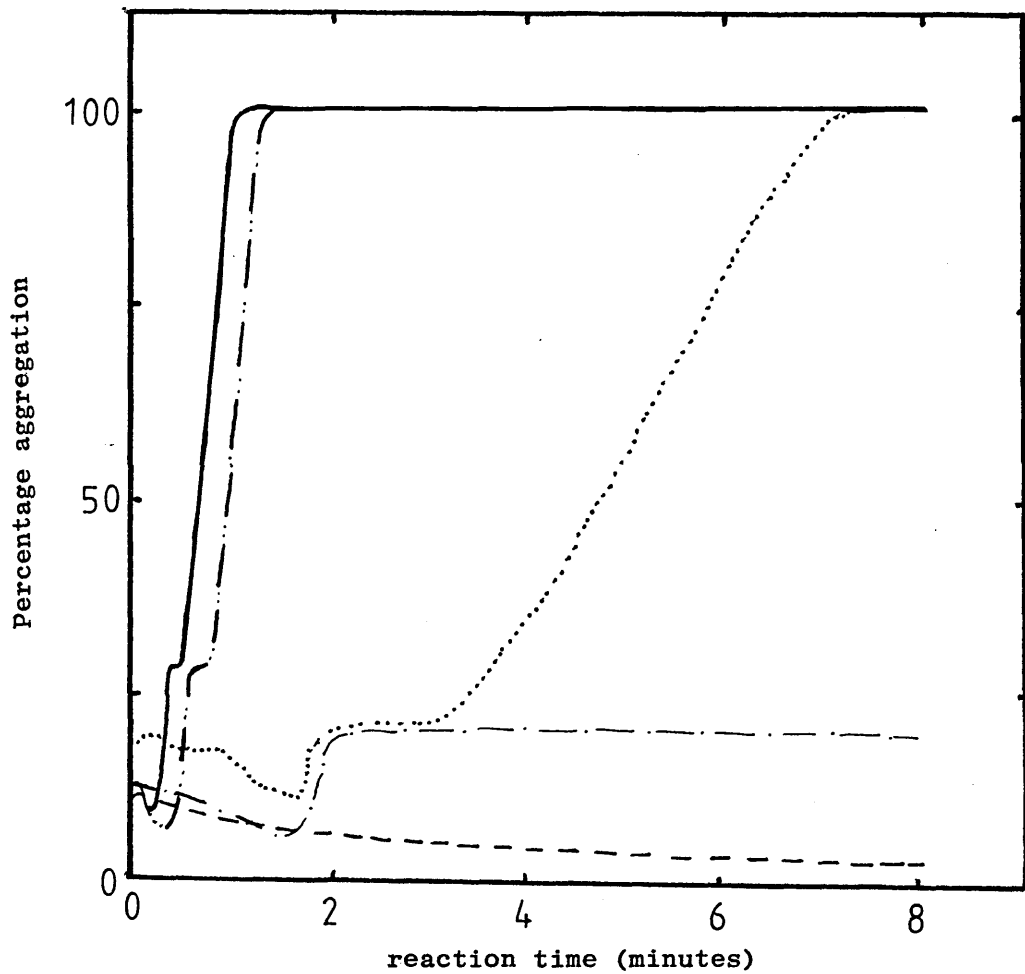


Alkali-modified λ -carrageenan was incubated with PRP for 3 minutes prior to the addition of thrombin (1.0 NIH U ml⁻¹) at zero time.

— control
---- λ -carrageenan (1.0 mg ml⁻¹)
-.-. λ -carrageenan (0.1 mg ml⁻¹)
..... λ -carrageenan (0.01 mg ml⁻¹)
- - - - λ -carrageenan (0.001 mg ml⁻¹)

Graph A.4.3.3

The effect of the 1_{Na} -carrageenan on thrombin-
induced platelet aggregation

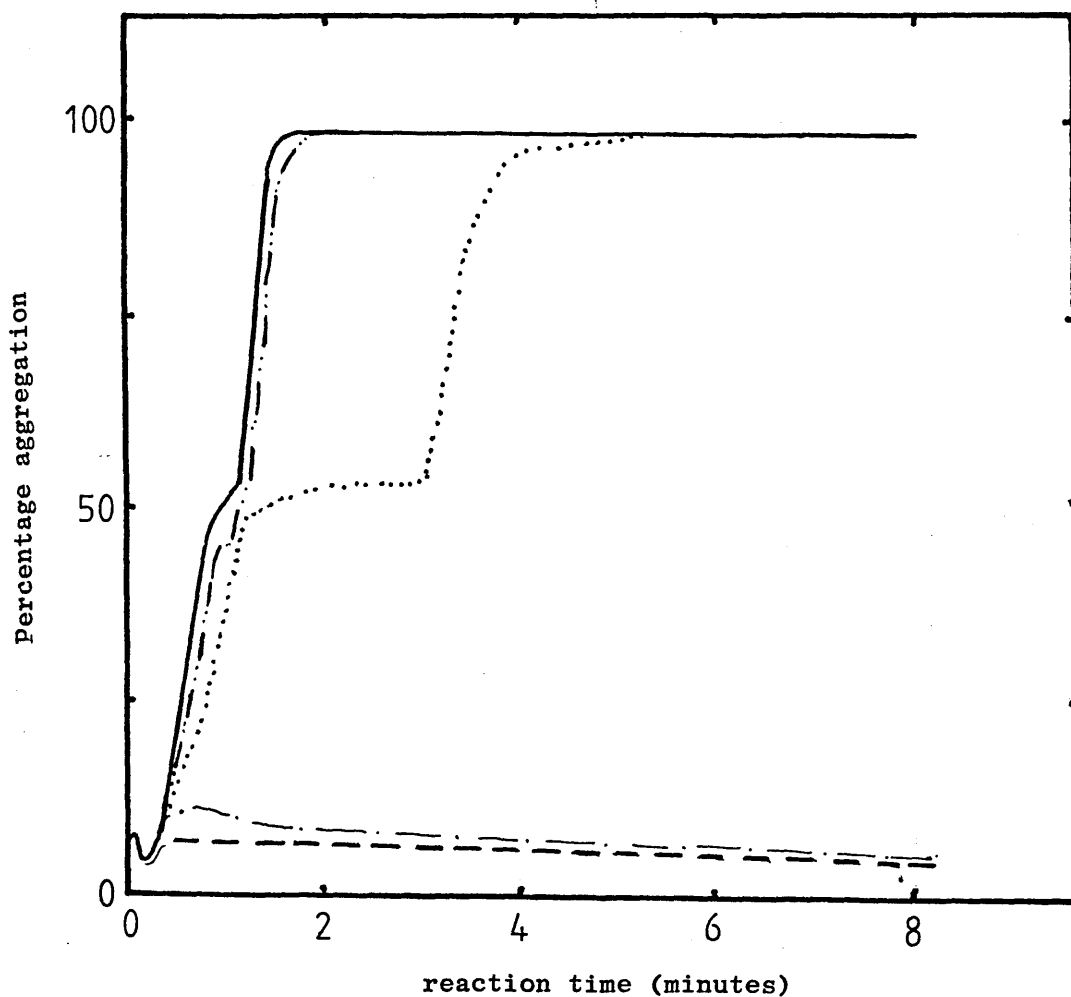


1_{Na} -Carrageenan was incubated with PRP for 3 minutes prior to the addition of thrombin ($1.0 \text{ NIH U ml}^{-1}$) at zero time.

————— control
 - - - - - 1_{Na} -carrageenan (1.0 mg ml^{-1})
 - . - . - 1_{Na} -carrageenan (0.1 mg ml^{-1})
 1_{Na} -carrageenan (0.01 mg ml^{-1})
 - - - - - 1_{Na} -carrageenan (0.001 mg ml^{-1})

Graph A.4.3.4

The effect of the lCa -carrageenan on thrombin-induced platelet aggregation

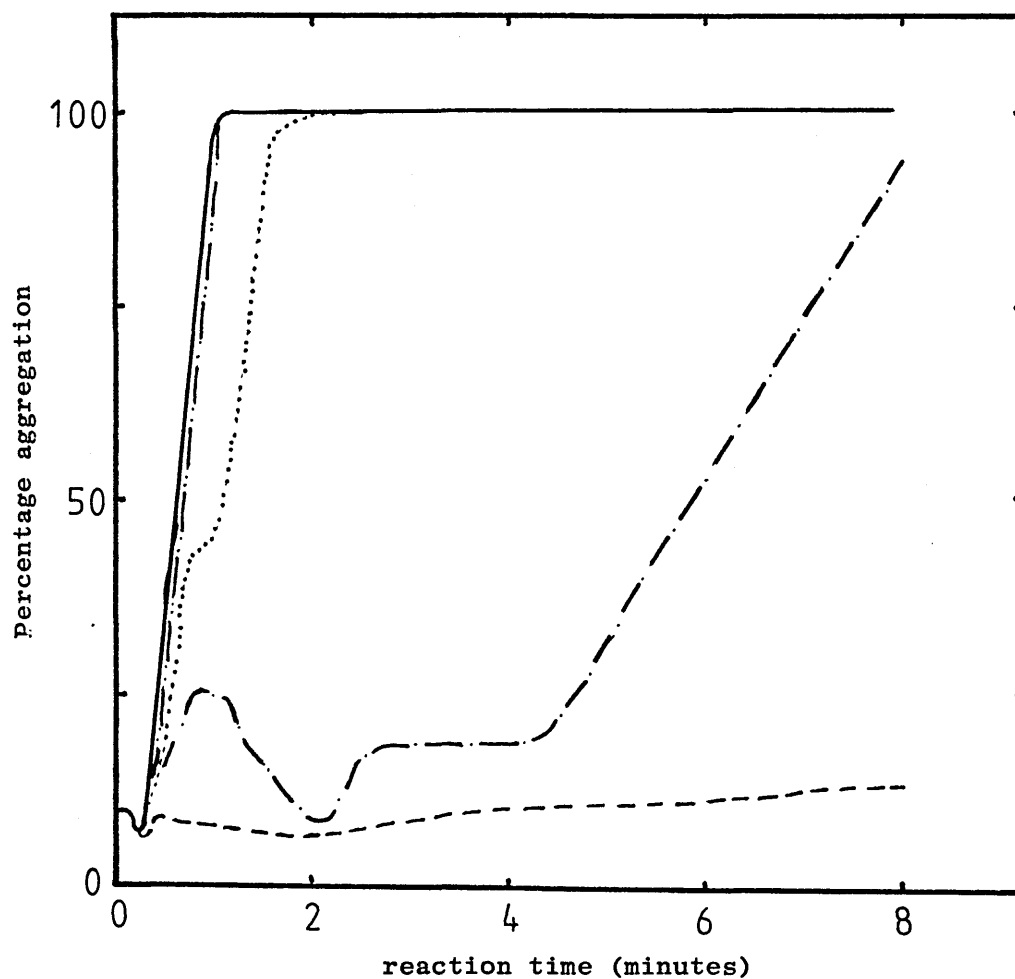


lCa -Carrageenan was incubated with PRP for 3 minutes prior to the addition of thrombin ($1.0 \text{ NIH U ml}^{-1}$) at zero time.

— control
 --- lCa -carrageenan (1.0 mg ml^{-1})
 - - - lCa -carrageenan (0.1 mg ml^{-1})
 lCa -carrageenan (0.01 mg ml^{-1})
 - · - · - lCa -carrageenan (0.001 mg ml^{-1})

Graph A.4.3.5

The effect of the κ_{Ca} -carrageenan on thrombin-
induced platelet aggregation

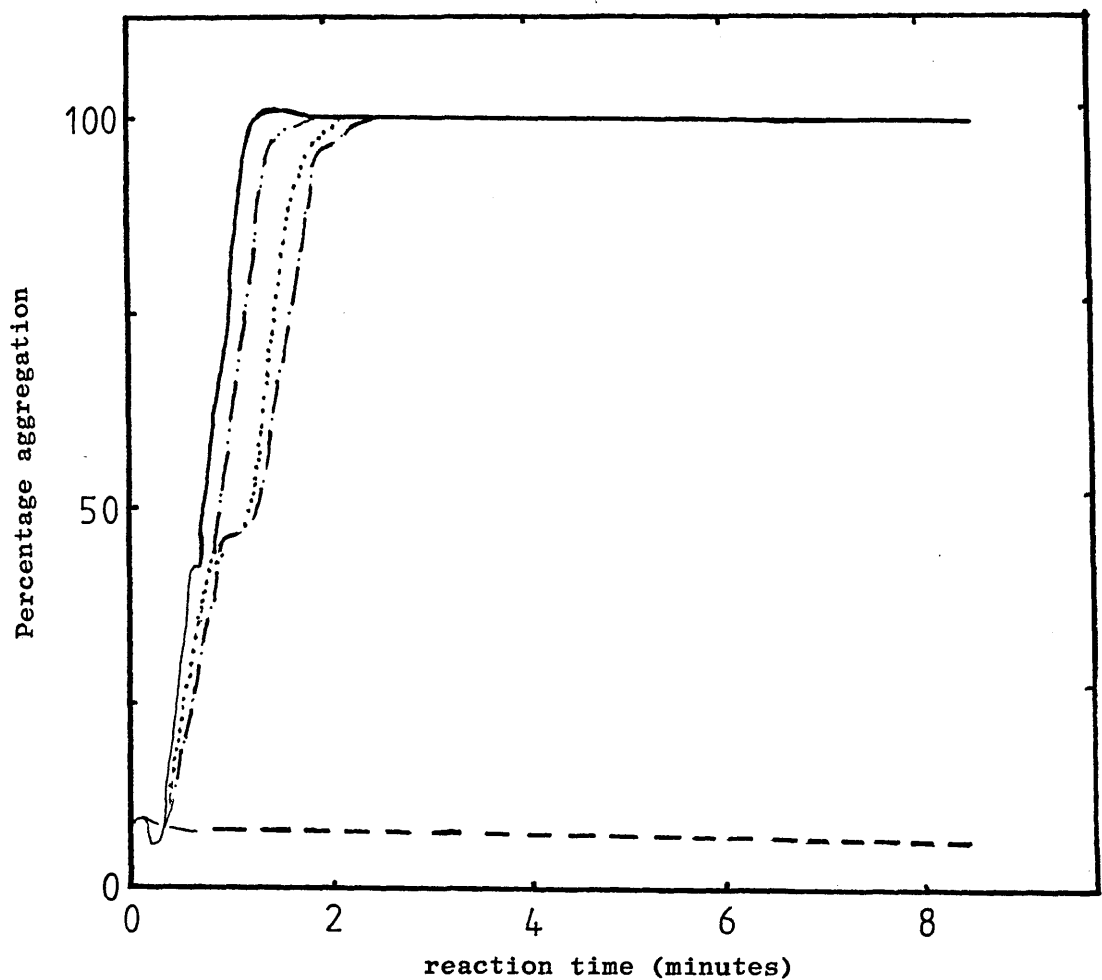


κ_{Ca} -Carrageenan was incubated with PRP for 3 minutes prior to the addition of thrombin (1.0 NIH U ml⁻¹) at zero time.

— control
 - - - κ_{Ca} -carrageenan (1.0 mg ml⁻¹)
 - · - κ_{Ca} -carrageenan (0.1 mg ml⁻¹)
 ····· κ_{Ca} -carrageenan (0.01 mg ml⁻¹)
 - - - κ_{Ca} -carrageenan (0.001 mg ml⁻¹)

Graph A.4.3.6

The effect of $\kappa\kappa$ -carrageenan on thrombin-
induced platelet aggregation



$\kappa\kappa$ -Carrageenan was incubated with PRP for 3 minutes prior to the addition of thrombin (1.0 NIH U ml⁻¹) at zero time.

— control
 - - - $\kappa\kappa$ -carrageenan (1.0 mg ml⁻¹)
 - · - $\kappa\kappa$ -carrageenan (0.1 mg ml⁻¹)
 · · · $\kappa\kappa$ -carrageenan (0.01 mg ml⁻¹)
 - - - $\kappa\kappa$ -carrageenan (0.001 mg ml⁻¹)

B. Ex vivo studies

Route and dose of carrageenan administered

Carrageenans were injected into animals via the intravenous, intraperitoneal and subcutaneous routes and assays performed on the blood samples subsequently collected in order to determine the optimum conditions for measurement of anticoagulant activity and toxicity effects.

Standard coagulation assays showed that the administration of carrageenan intraperitoneally resulted in uptake of the carrageenan, and produced an anticoagulant effect without the extensive disseminated intravascular coagulation found when the compounds were administered intravenously.

Extrapolation of the effective anticoagulant carrageenan concentration found in the in vitro assays to the ex vivo test system resulted in a range of concentrations between 10 and 60 mg kg⁻¹ at 5 mg intervals being tested. Further tests resulted in a concentration of 20 mg kg⁻¹ being used. In each of the ex vivo tests performed, K-carrageenans had little or no effect, while the most active carrageenan fractions were the alkali-modified λ - and λ_{Na} -carrageenan. Therefore, all further studies were carried out using these two carrageenans, administered via the peritoneal route, at a concentration of 20 mg kg⁻¹.

B.1 Blood clotting assay

B.1.1 Partial thromboplastin clotting time assay

PTT assays were carried out on plasma obtained from carrageenan-treated animals. No less than five animals were used per group.

Results illustrated in Graph B.1.1.1, p. 109 indicate that maximal inhibition of clotting occurred at 12 hours post dose for both alkali-modified λ - and ι -carrageenan. The PTT values of samples taken at 24 hours after administration of carrageenan were similar to control values. An initial peak was observed 2 hours after the administration of carrageenan.

B.1.2 Russell's viper venom clotting time

The results obtained for the inactivation of Factor X illustrate a significant anticoagulant effect with alkali-modified λ -carrageenan over the 2-12 hour period while little or no activity was observed with ι -carrageenan (Graph B.1.2.1, p. 110).

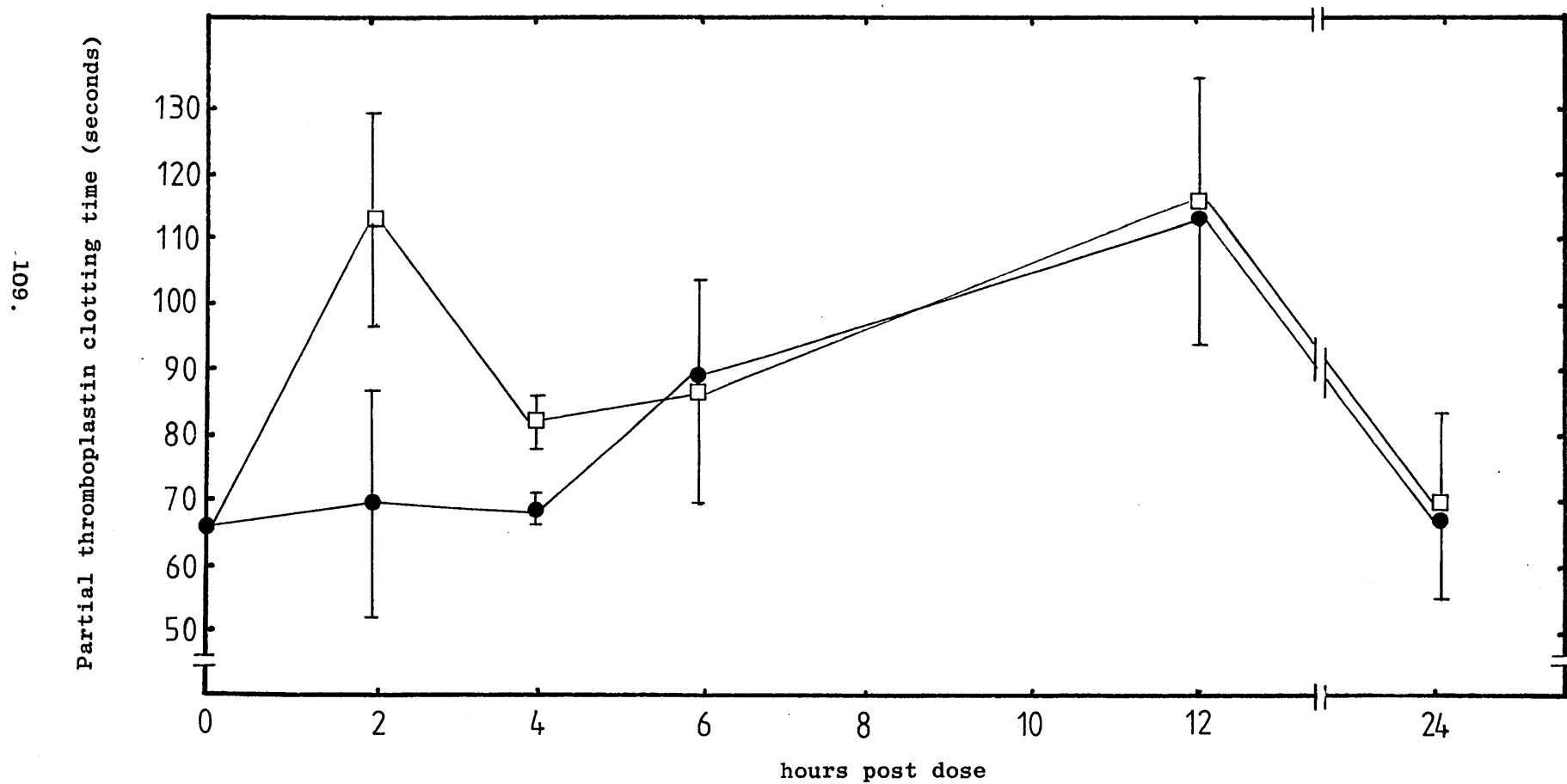
Comparison of alkali-modified λ -carrageenan effects on results of PTT and RVV assays shows a shift of greatest anticoagulant activity from 12 hours to 6 hours post dose respectively.

B.1.3 Thrombin clotting time assay

Both carrageenans were observed to be anticoagulant, with λ -carrageenan activity potentiated at 4 hours and ι -carrageenan maximal at 6-12 hours (Graph B.1.3.1, p.111). A value of infinity was obtained for each of seven alkali-modified λ -carrageenan treated animals. As before, these results are taken from triplicate readings per animal with standard errors below 8.2 per cent.

Graph B.1.1.1

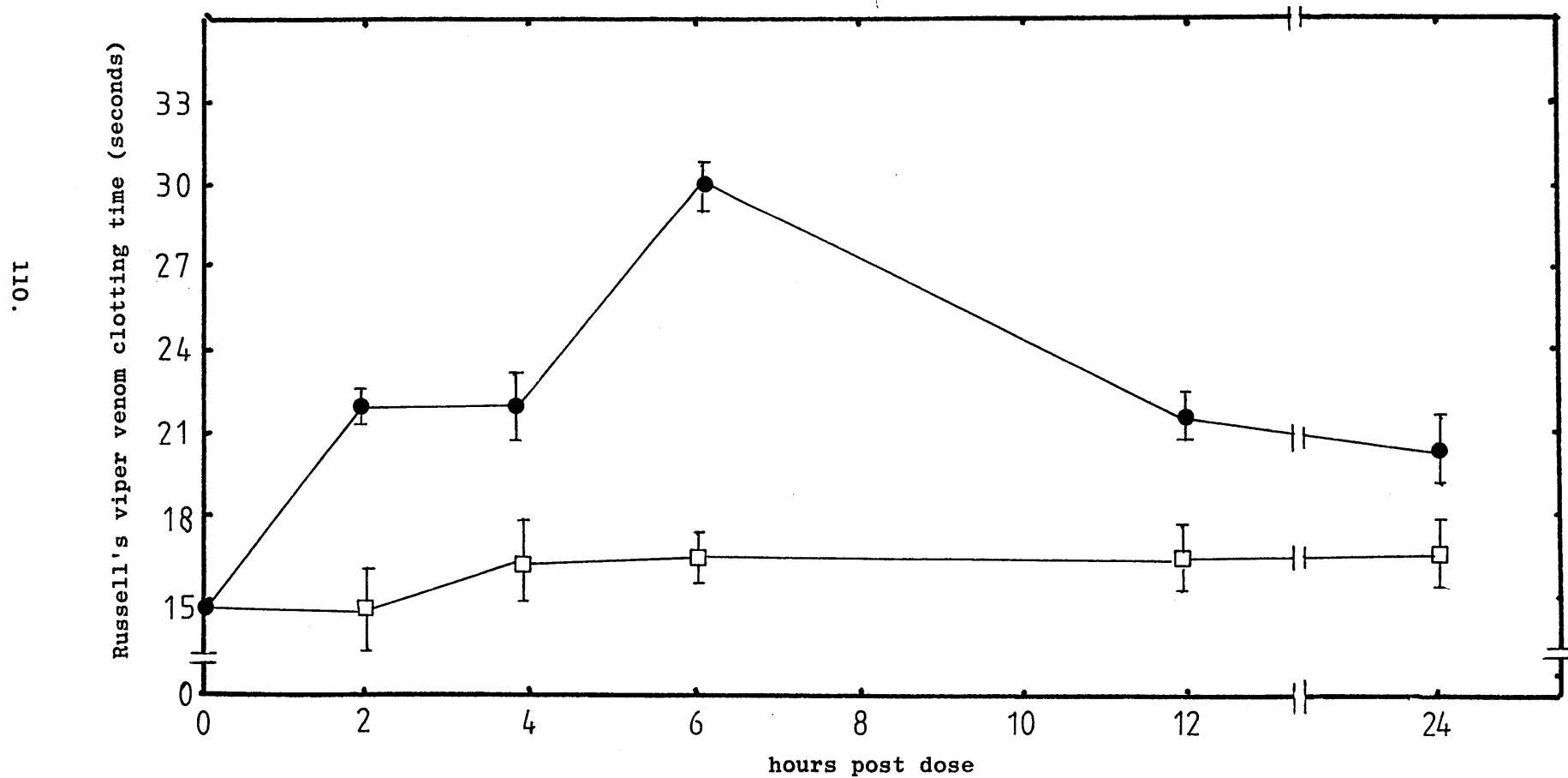
The effect of alkali-modified λ - and ι -carrageenan on the partial thromboplastin
clotting time of plasma



Effect of alkali-modified λ -carrageenan (●) and ι -carrageenan (□) ex vivo on the PTT clotting time of plasma. Control time of 64.8 ± 5.2 s when $n > 15$.

Graph B.1.2.1.

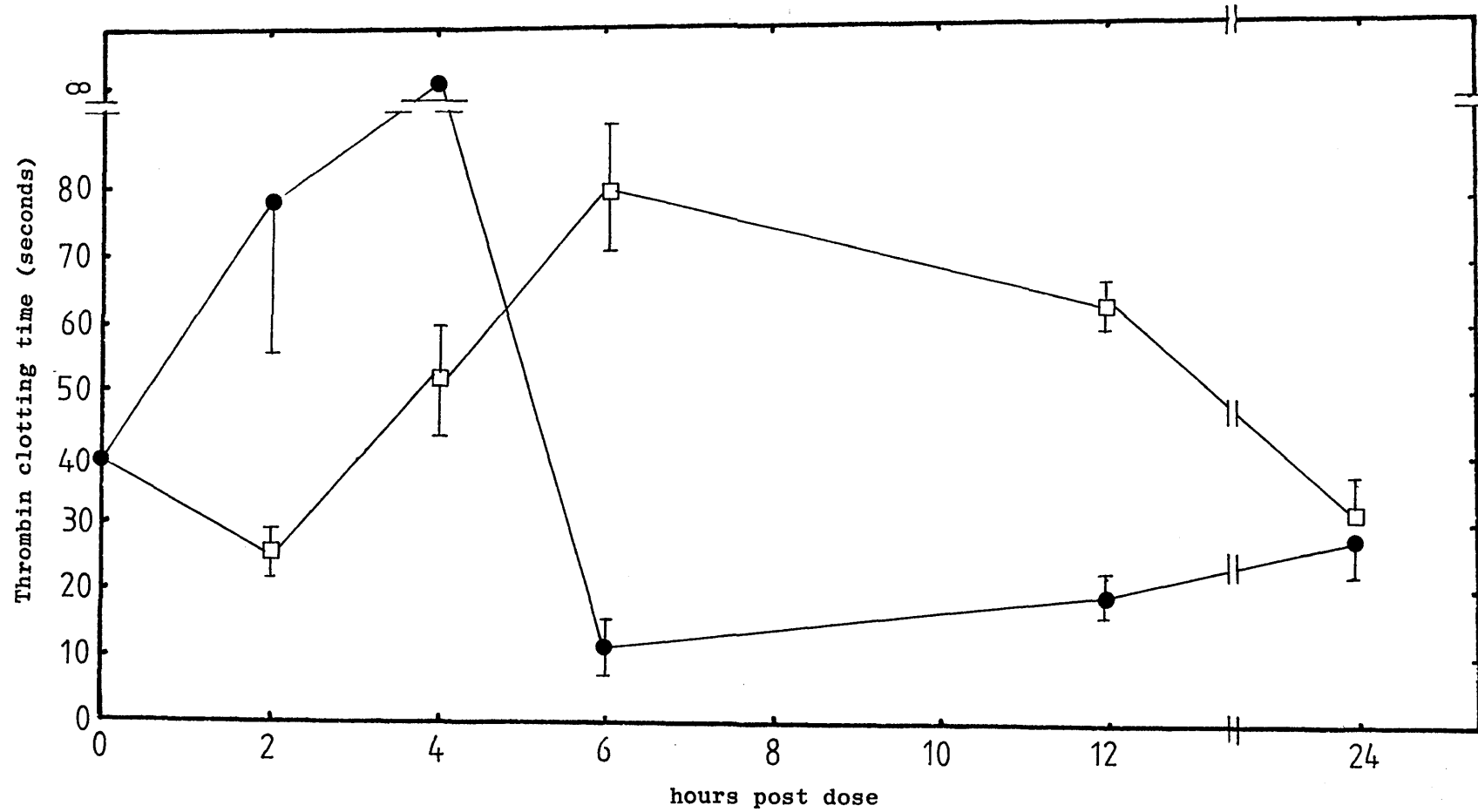
The effect of alkali-modified λ - and ι -carrageenan on Russell's viper venom
clotting time of plasma



Effect of alkali-modified λ - (●) and ι -carrageenan (□) on RVV clotting time of plasma. Control time was 15.2 ± 1.6 s when $n > 15$.

Graph B.1.3.1

The effect of alkali-modified λ - and ι -carrageenan on the thrombin clotting time of plasma



Effect of alkali-modified λ -carrageenan (●) and ι -carrageenan (□) on the thrombin clotting time of plasma. Control time was 40.1 ± 4.2 s when $n > 15$.

Comparison of PTT assay results with thrombin clotting assay results suggests that, in the case of alkali-modified λ -carrageenan, onset of anticoagulant activity occurs earlier (at 4 hours compared to 12 hours). In the case of λ -carrageenan, the antithrombotic effect is sustained over the 12 hour period, returning to control value at 24 hours.

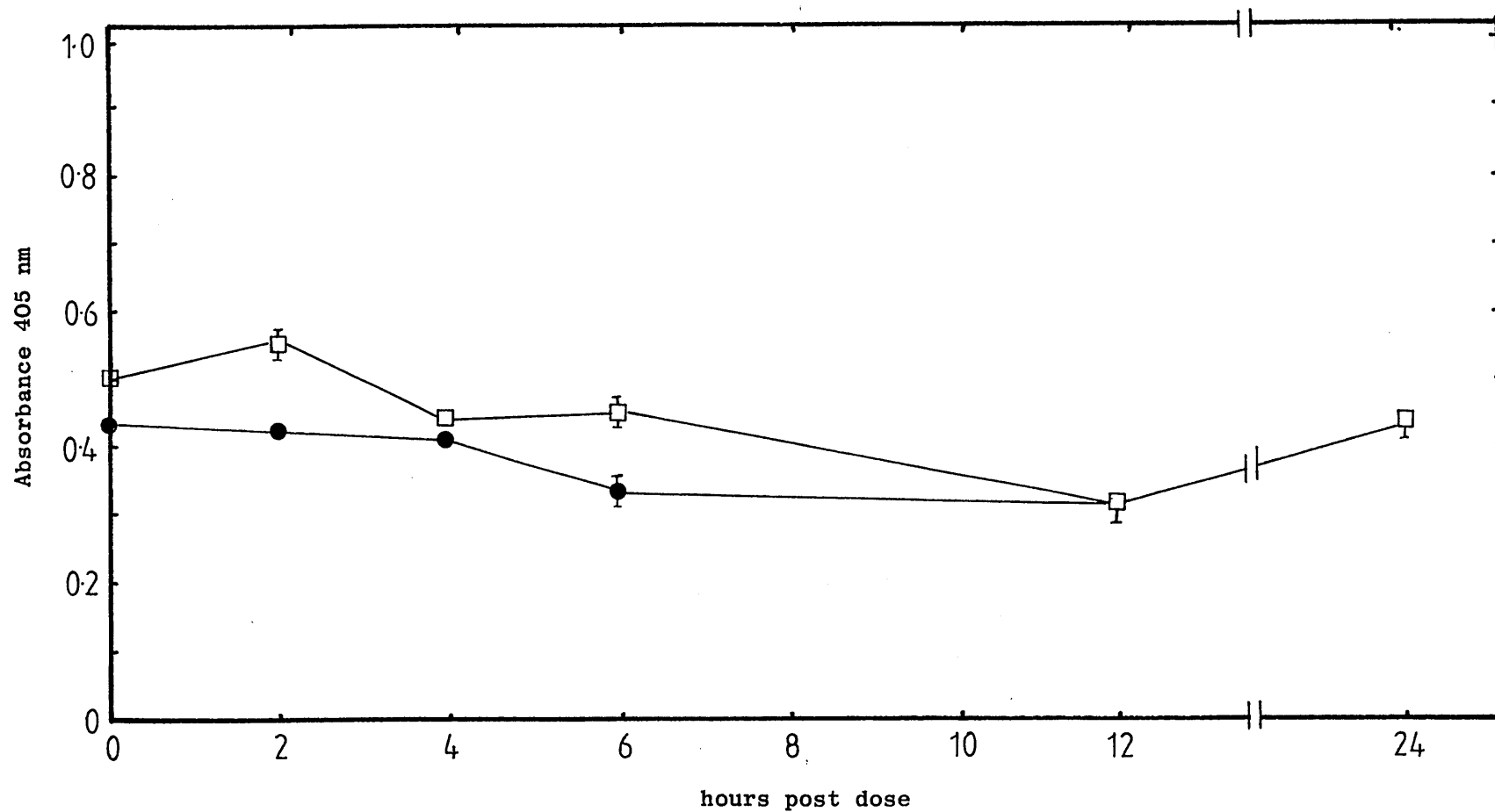
B.1.4 Amidolytic activity of Factor X (Factor Xa)

Results were obtained for Factor X (Factor Xa) activation shown by the amidolytic activity of chromogenic substrate S2222 (Methods section 1.2.1). The alkali-modified λ -carrageenan fraction was found to inhibit Factor X activation between 6-12 hours, while λ -carrageenan exhibited little or no inhibition of Factor X (Graph B.1.4.1, p. 113).

The results obtained correlate with those of the functional assay using Russell's viper venom, although the effect of carrageenan on Factor X directly is shown to be less significant using the amidolytic assay.

Graph B.1.4.1

The effect of alkali-modified λ - and ι -carrageenan on Factor X (Factor Xa) amidolytic activity



Effect of alkali-modified λ -carrageenan (●) and ι -carrageenan (□) on Factor X (Factor Xa) amidolytic activity in plasma. Control value was 0.50 ± 0.02 nm when $n > 5$.

B.2 Fibrinolysis

B.2.1 Euglobulin derived fibrinolytic activity

A euglobulin precipitate was obtained by acidification of plasma obtained from carrageenan-treated animals. As before, no less than five animals per group were assayed, and each euglobulin was tested in duplicate on fibrin plates. Results were expressed as the diameter of areas of lysis obtained from two perpendicular axes, with values less than the control indicating inhibition of fibrinolysis.

It was observed that both carrageenans inhibited fibrinolysis (Graph B.2.1.1, p.115). The ι -carrageenan-treated animals showed inhibition at 2 hours while alkali-modified λ -carrageenan-treated animals inhibited fibrinolysis between 2 and 6 hours, greatest inhibition occurring at 6 hours.

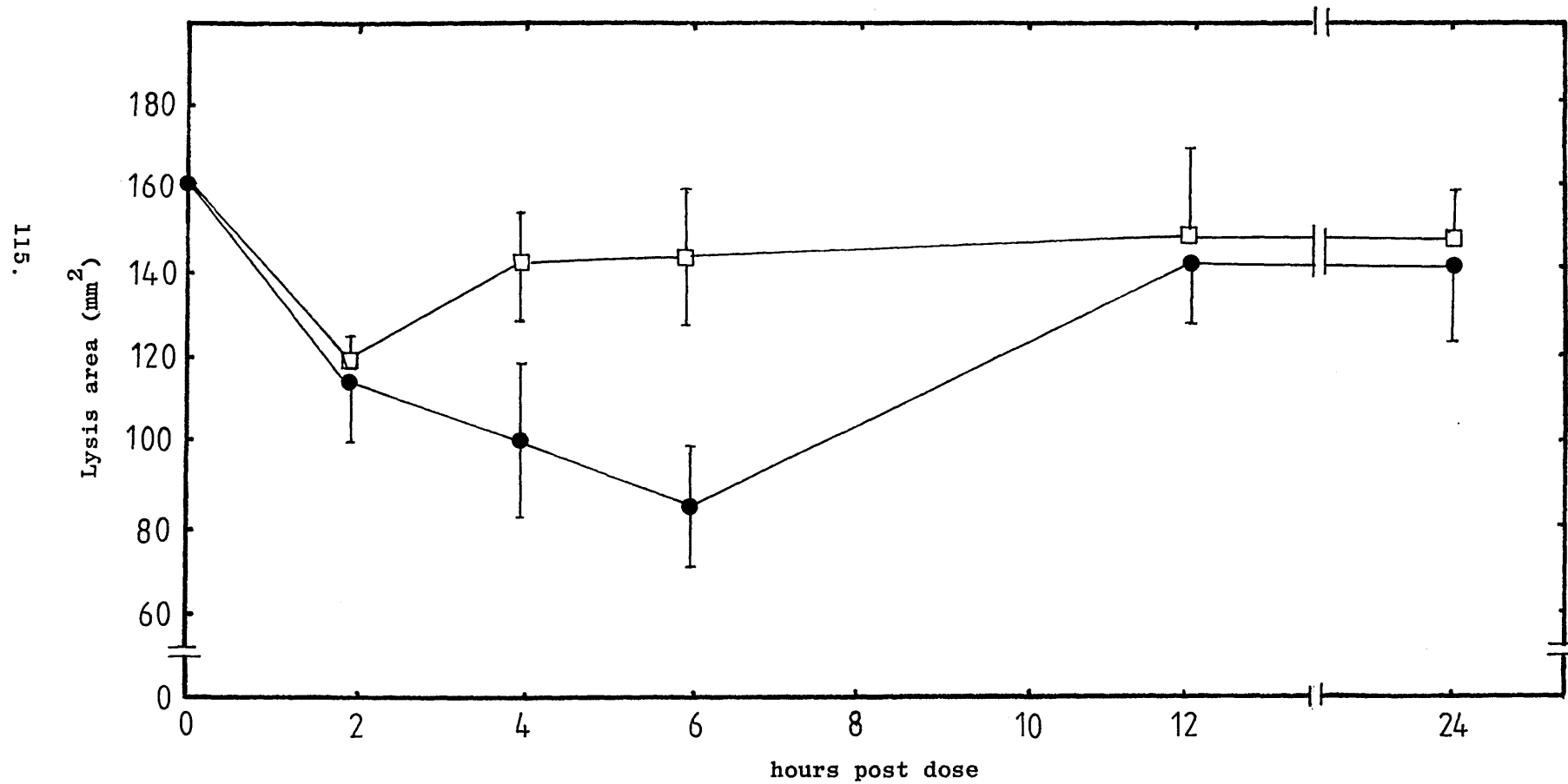
B.2.2 Inhibitor studies with ϵ -aminocaproic acid

Plasma obtained from carrageenan-treated animals was incubated with ϵ -ACA (2 mM) prior to the formation of the euglobulin precipitate. As previously stated, duplicate assays were carried out on each euglobulin fraction.

ι -Carrageenan produced no significant difference between control and test values (Graph B.2.2.1, p.116). The plasma euglobulin derived from alkali-modified λ -carrageenan treated animals was inhibitory to fibrinolysis over the 2-4 hour period with no statistically significant difference at 6 hours.

Graph B.2.1.1

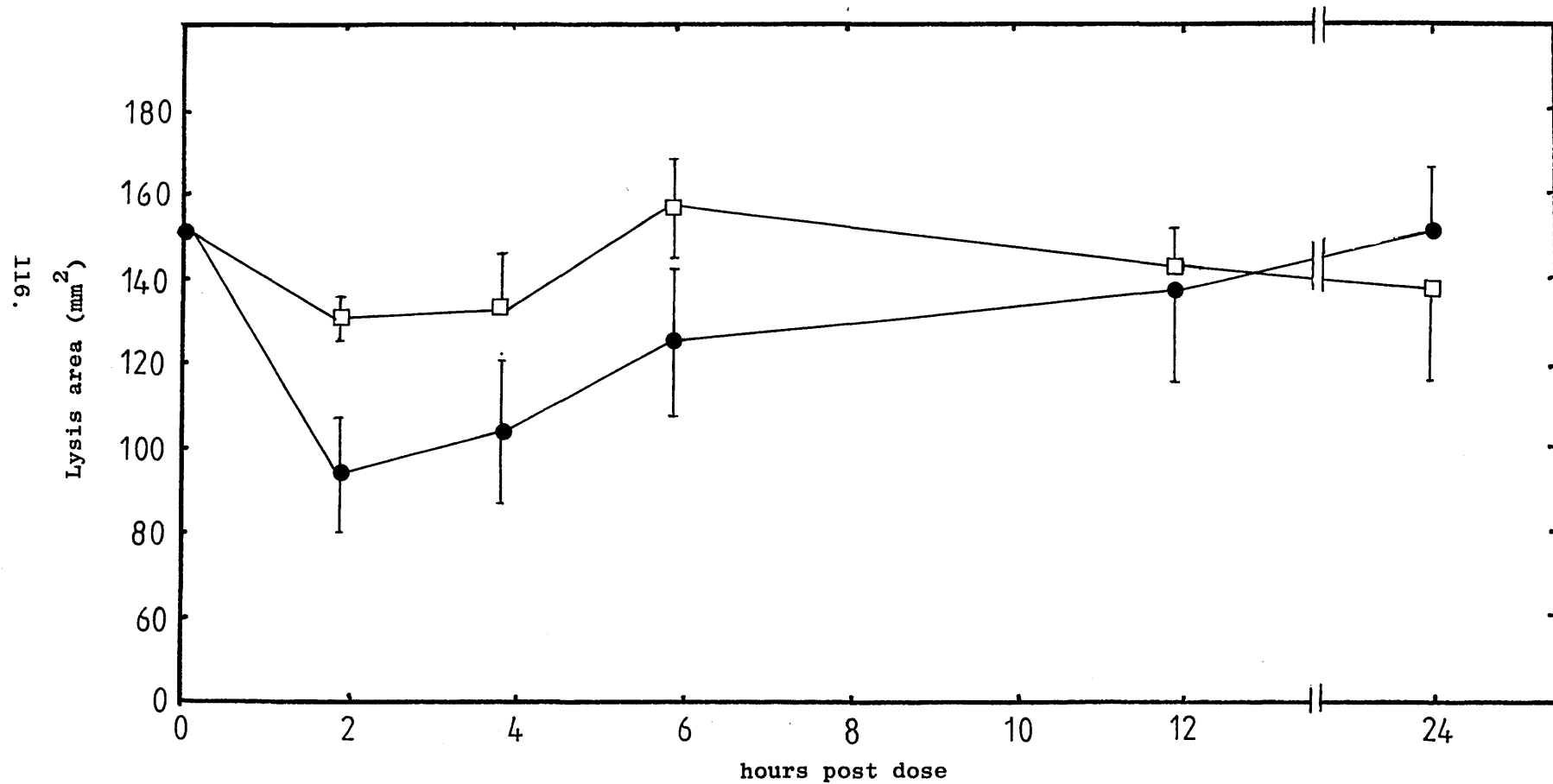
The effect of alkali-modified λ - and ι -carrageenan on plasma euglobulin
fibrinolytic activity



Effect of alkali-modified λ -carrageenan (●) and ι -carrageenan (□) on euglobulin-derived fibrinolytic activity. Control value was $160.0 \pm 16.0 \text{ mm}^2$ when $n > 5$.

Graph B.2.2.1

The effect of alkali-modified λ - and ι -carrageenan on euglobulin-derived
fibrinolytic activity in the presence of ϵ -aminocaproic acid



Effect of alkali-modified λ -carrageenan (●) and ι -carrageenan (□) on euglobulin-derived fibrinolytic activity in the presence of ϵ -ACA (2 mM). Control value was $153.0 \pm 17.5 \text{ mm}^2$ when $n > 5$.

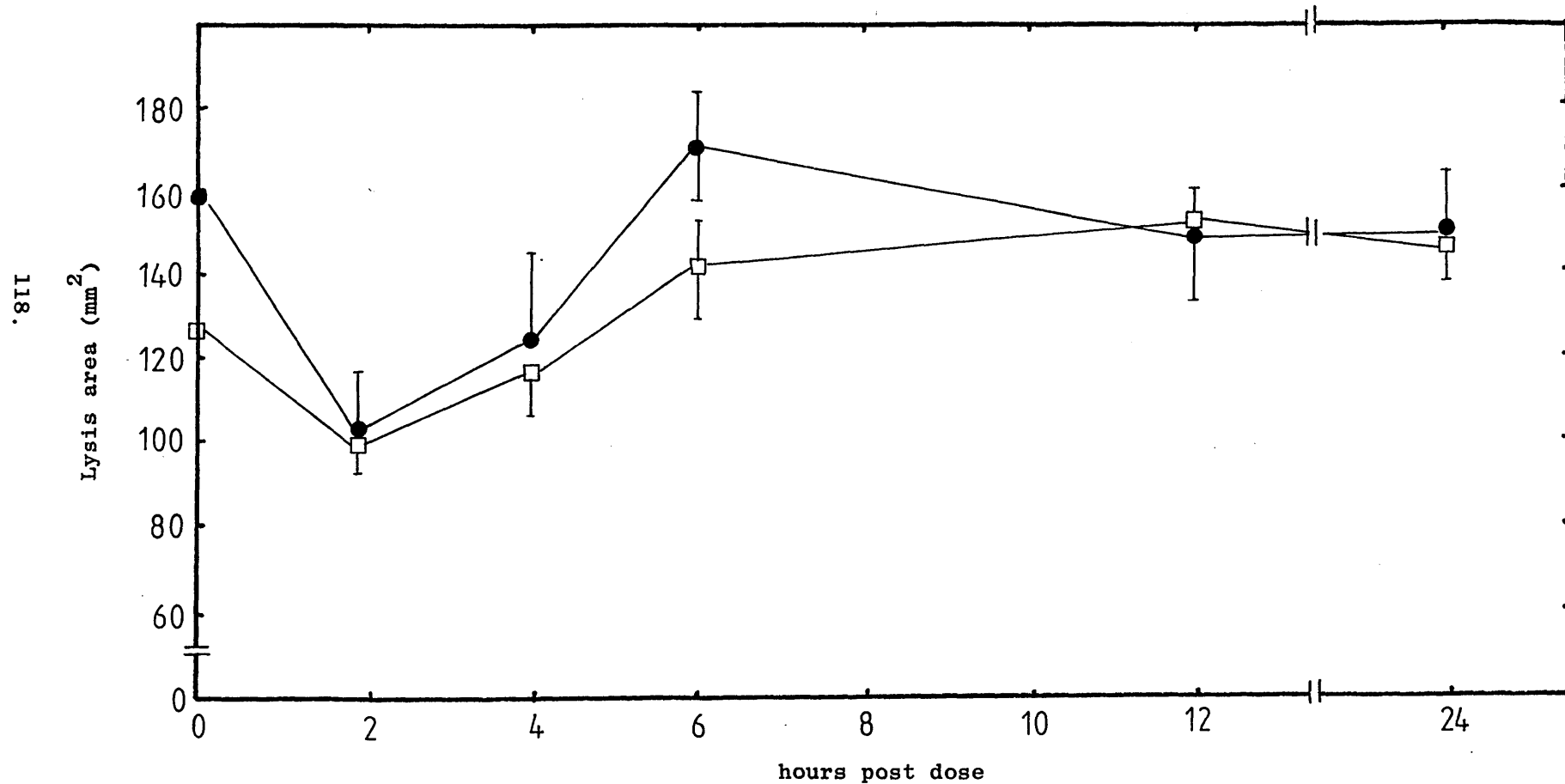
B.2.3 Inhibitor studies with flufenamic acid

The incorporation of flufenamic acid into carrageenan-treated plasma prior to euglobulin precipitation resulted in inhibition of fibrinolysis peaking at 2 hours, returning to control fibrinolytic activity at 4 hours (Graph B.2.3.1, p. 118).

Comparison with normal fibrinolytic activity showed that flufenamic acid had little or no effect on ι -carrageenan-treated plasma, whereas the activity of alkali-modified λ -carrageenan was reduced.

Graph B.2.3.1

The effect of alkali-modified λ - and ι -carrageenan on euglobulin-derived fibrinolytic activity in the presence of flufenamic acid



Effect of alkali-modified λ -carrageenan (●) and ι -carrageenan (□) on euglobulin-derived fibrinolytic activity in the presence of flufenamic acid (12 mM). Control value was 150.0 ± 20.0 mm² when $n > 5$.

B.3 Kallikrein-kinin system

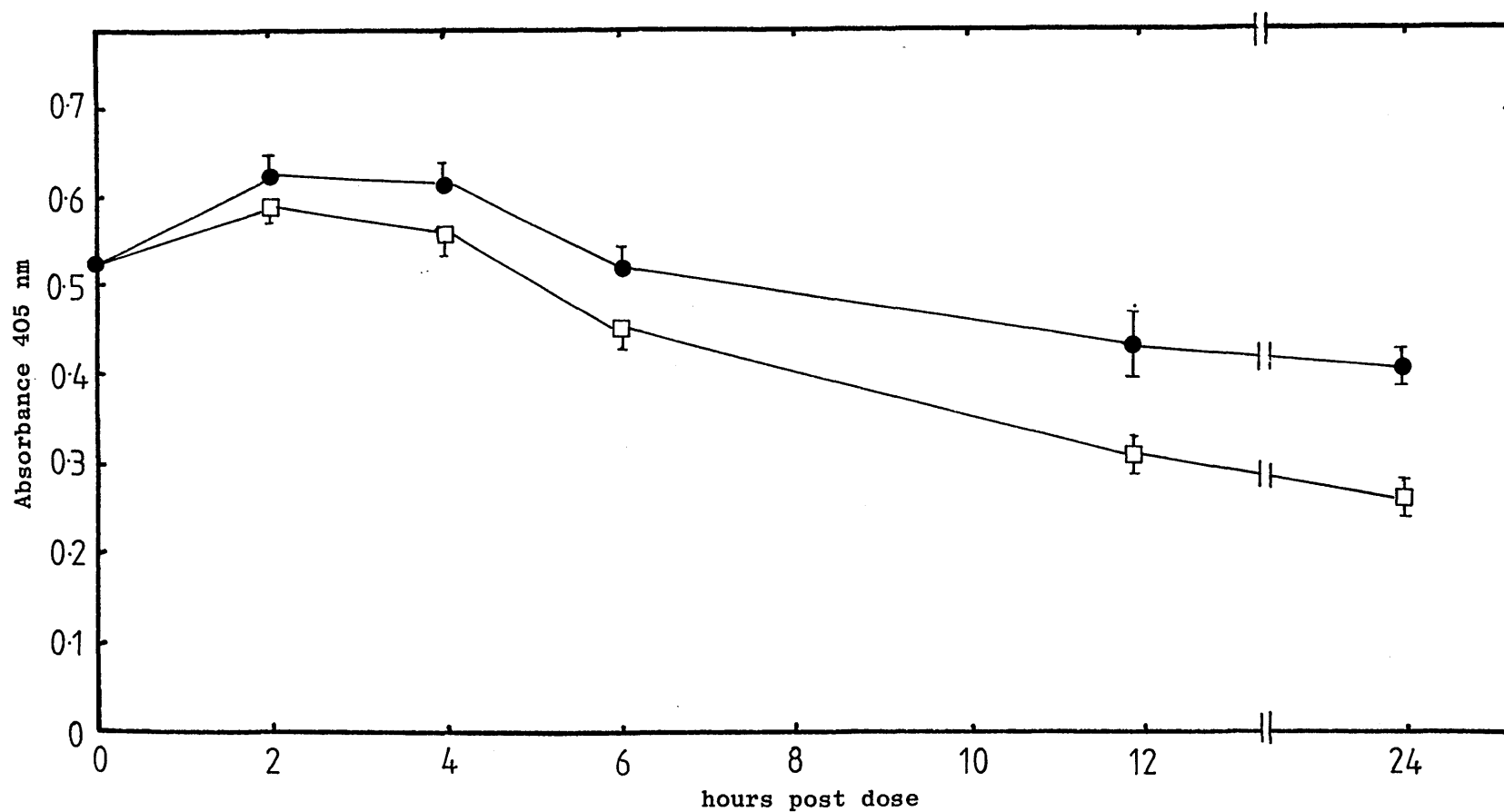
B.3.1 Amidolytic assay for carrageenan derived prekallikrein activation

To observe the effect of carrageenan on the activation of plasma prekallikrein, plasma obtained from carrageenan-treated animals was assayed using the chromogenic substrate S2302. The conversion of prekallikrein to the proteinase kallikrein resulted in the release of pNA from the substrate and the resultant colour change was measured spectrophotometrically.

Administration of carrageenan resulted in increased amidolysis over the initial 4 hour period, but inhibition over 12-24 hours (Graph B.3.1.1, p. 120). The plasma of alkali-modified λ -carrageenan-treated animals showed greater amidolysis than that of ι -carrageenan but less inhibition over the 12-24 hour period. The prekallikrein-catalysed amidolytic rates observed in plasma samples taken from both groups of animals returned to normal levels within 36 hours.

Graph B.3.1.1

The effect of alkali-modified λ - and ι -carrageenan on prekallikrein



Effect of alkali-modified λ -carrageenan (●) and ι -carrageenan (□) on prekallikrein using chromogenic substrate S2302. Control absorbance was 0.5 ± 0.01 when $n > 5$.

B.4 Platelet studies

B.4.1 Platelet counts in platelet-rich plasma

Platelet counts were carried out on PRP samples. Results expressed in Graph B.4.1.1 (p. 122) illustrate the mean and standard errors for no less than five animals per group with a control value of 2.7×10^5 platelets $\text{mm}^{-3} \pm 0.2 \times 10^5$. Standard errors were within the range 2-19 per cent.

Both alkali-modified λ - and ι -carrageenan suppressed the platelet count, with only ι -carrageenan-treated animals returning to normal at 36 hours post dose.

B.4.2 Adenosine 5'-diphosphate-induced aggregation

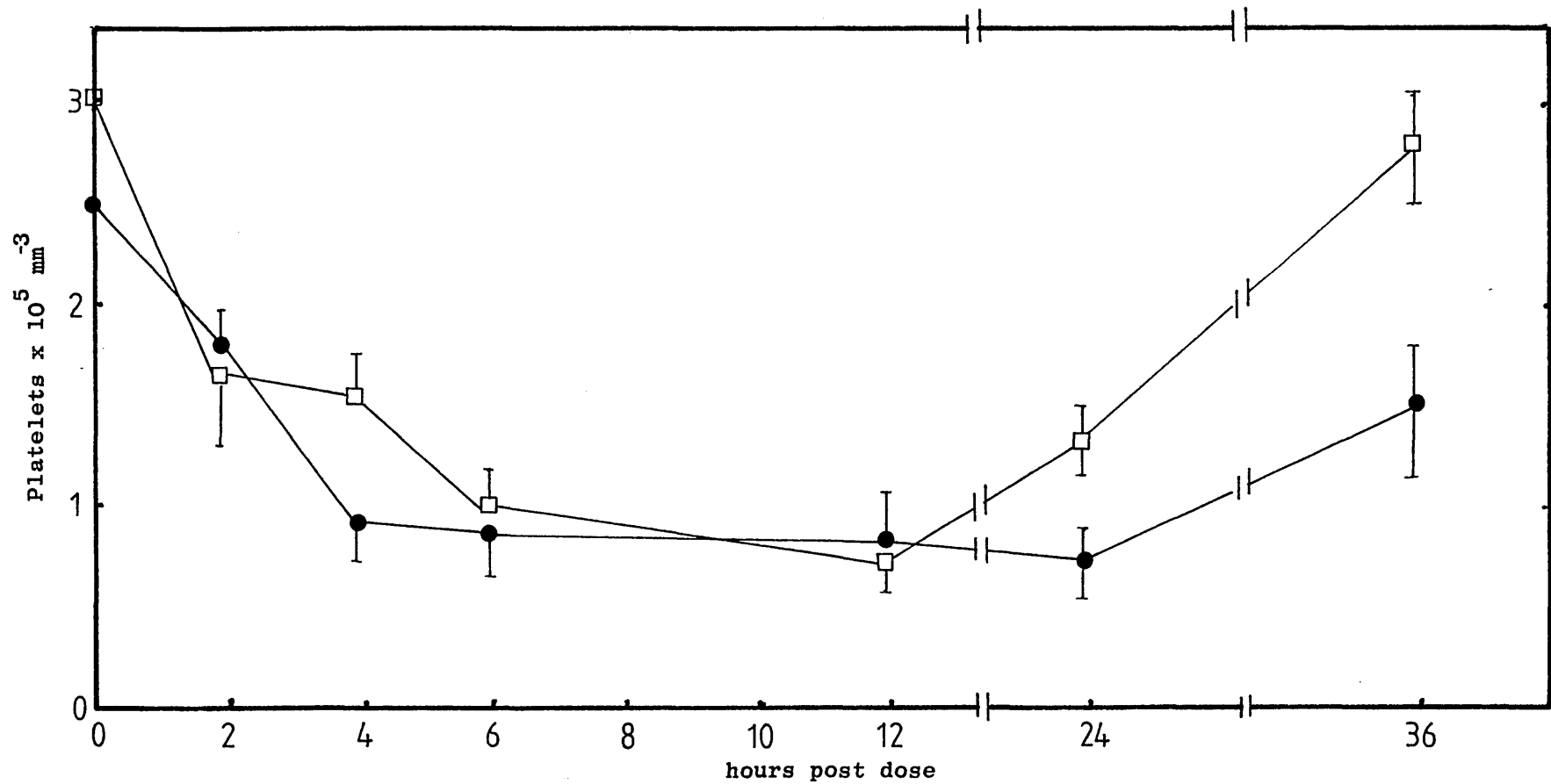
Platelet aggregation was carried out as previously described (Methods Section 4.2). As before, no less than five animals per group were used. The ADP activity was assessed using three concentrations on duplicate platelet samples.

Graph B.4.2.1(a) (p. 123) illustrates a typical control aggregation response while Graphs B.4.2.1(b) and (c) (p. 123) show the influence of ι - and alkali-modified λ -carrageenan respectively on ADP-induced aggregation 4 hours post dose. In all cases first phase reversible aggregation was observed.

Results of aggregation induced by $1.0 \mu\text{M}$ ADP indicated a control value of $58. \pm 4.4$ per cent. Aggregation values below 58 per cent indicate inhibition. It was observed that over a time period of 2-36 hours alkali-modified λ -carrageenan significantly inhibited ADP-induced aggregation, reducing the ADP response to 6.4 per cent of normal (Graph B.4.2.2, p. 124). ι -Carrageenan also caused

Graph B.4.1.1

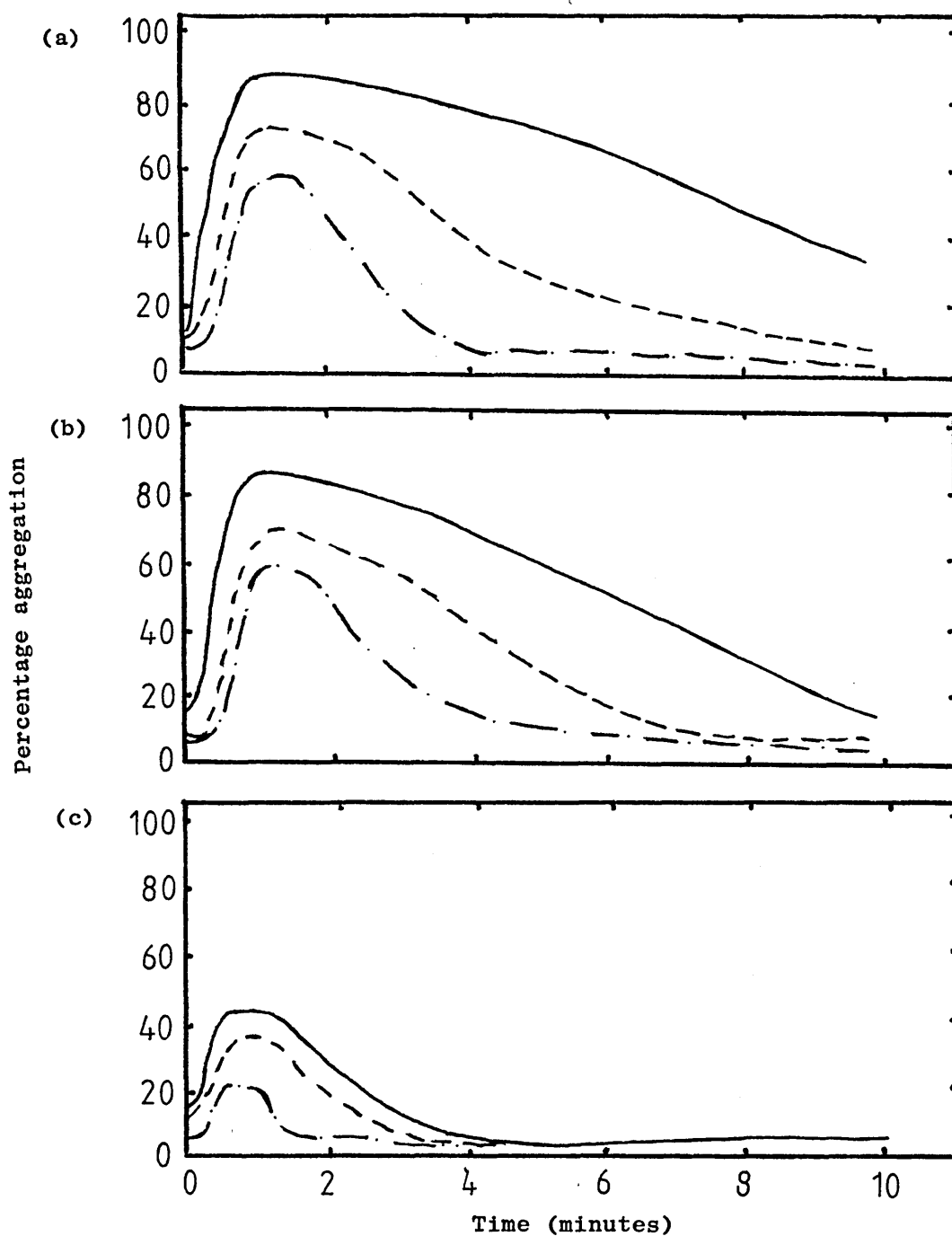
The effect of alkali-modified λ - and ι -carrageenan on platelet counts



The effect of alkali-modified λ -carrageenan (●) and ι -carrageenan (□) on platelet counts. Control platelet count of $2.7 \pm 0.2 \times 10^5 \text{ mm}^{-3}$ when $n > 5$.

Graph B.4.2.1

Aggregation responses to ADP at 4 hours post dose

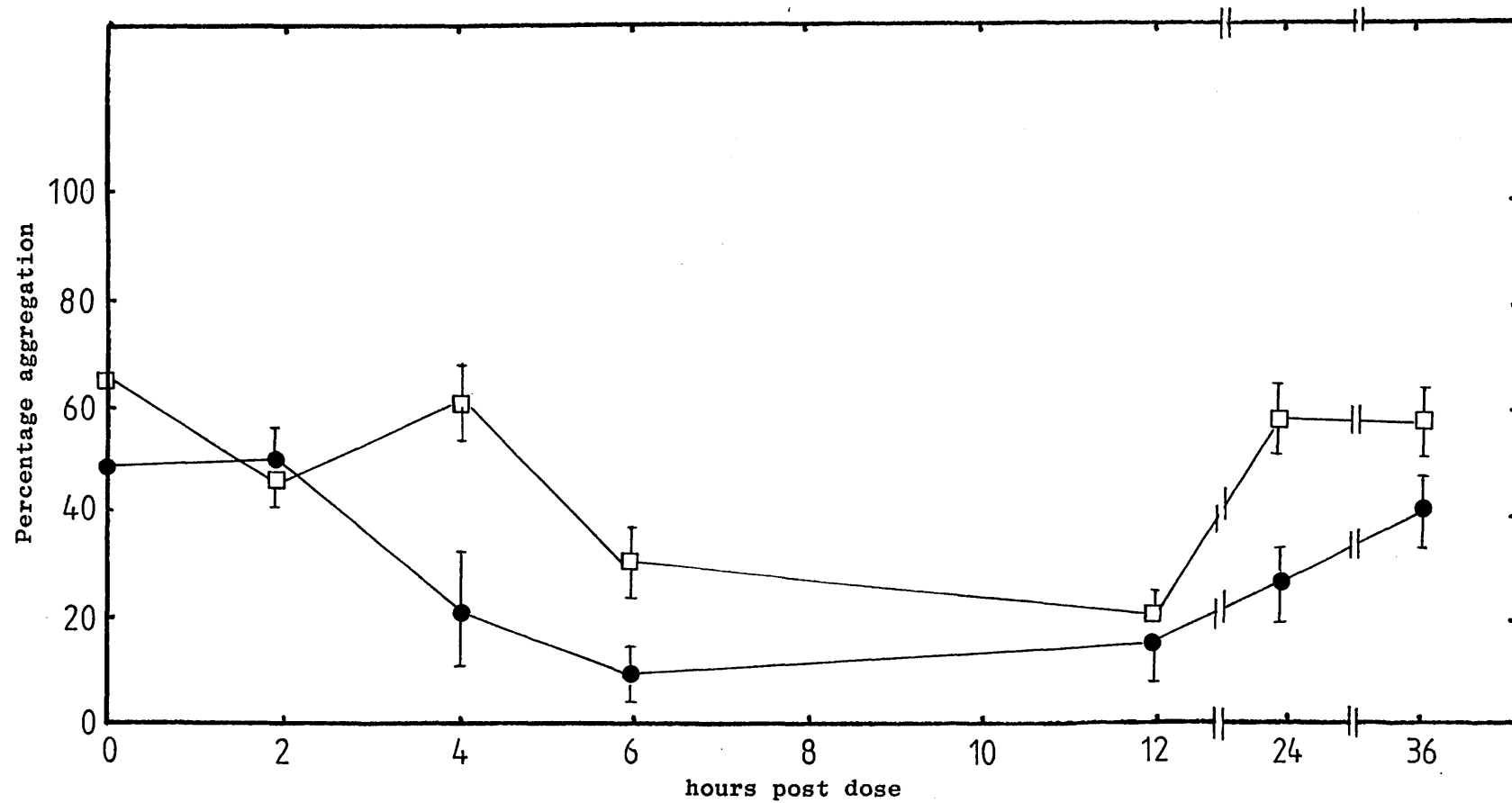


Effect of carrageenan on ADP induced aggregation at 4 hours
(a) normal response (b) 1-carrageenan (c) alkali-modified λ -carrageenan.

— 50 μ M ADP
- - - 10 μ M ADP
- . - 1.0 μ M ADP

Graph B.4.2.2

The effect of alkali-modified λ - and ι -carrageenan on ADP induced platelet aggregation



Effect of alkali-modified λ -carrageenan(●) and ι -carrageenan (□) on platelet responsiveness to ADP (1.0 μ M). Control value was 58 ± 4.4 per cent when $n > 5$.

inhibition of ADP-induced aggregation. However, in this case, onset of inhibition occurred at 6 hours and the response to ADP treatment returned to normal at 24 hours.

At an ADP concentration of 10 μ M, a control value of 72.5 ± 6.4 per cent was observed (Graph B.4.2.3, p. 126). At lower ADP concentrations, alkali-modified λ -carrageenan inhibited ADP-induced aggregation but to a lesser degree in both extent and duration of platelet inhibition at 6-12 hours. A small peak was observed at 4 hours.

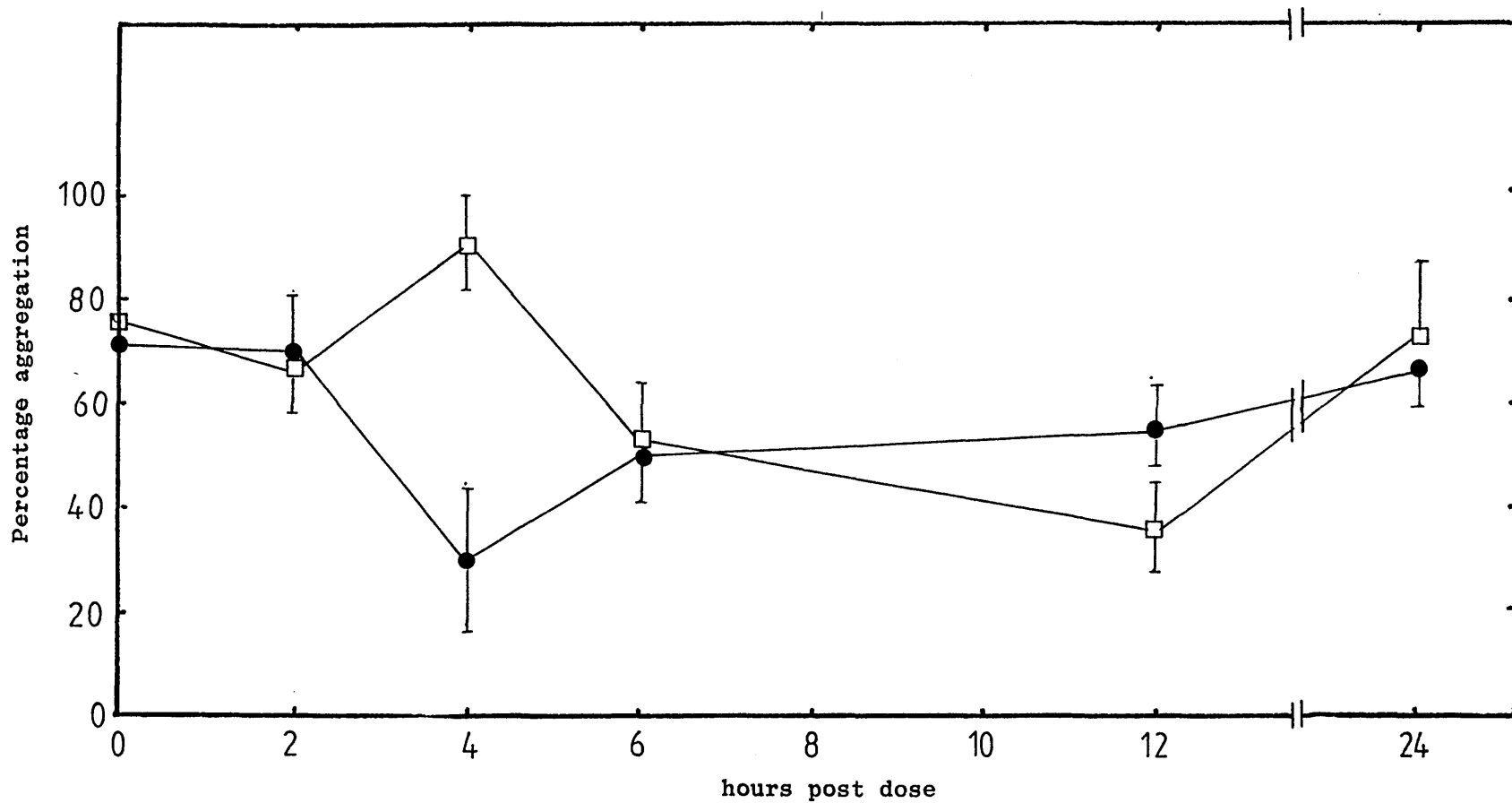
A final concentration of 50 μ M ADP resulted in a control value of 82.5 ± 5.2 per cent aggregation. The aggregation response was still reversible, showing no indication of second phase aggregation even when platelets were left stirring at 900 rpm for 30 minutes. Results illustrated on Graph B.4.2.4 (p. 127) suggested that both carrageenans exerted an antiplatelet effect with respect to ADP-induced platelet aggregation. As before with the functional clotting assays, λ -carrageenan exhibited a delay of 6 hours prior to onset of an inhibitory response. The extent of inhibition during the 6-12 hour period was significant. The alkali-modified λ -carrageenan showed an inhibitory response, peaking at 4 hours and returning to normal within the 12-24 hour period.

B.4.3 Thrombin-induced aggregation

The influence of thrombin on the platelets of carrageenan-treated animals was assayed by the addition of thrombin to PRP and measurement of the aggregation response over a 10 minute period. Graph B.4.3.1(a) (p. 128) illustrates a typical thrombin-induced response in saline-treated animals, and Graphs B.4.3.1(b) and (c) (p. 128) show the influence of λ - and alkali-modified λ -carrageenan respectively at 4 hours post dose.

Graph B.4.2.3

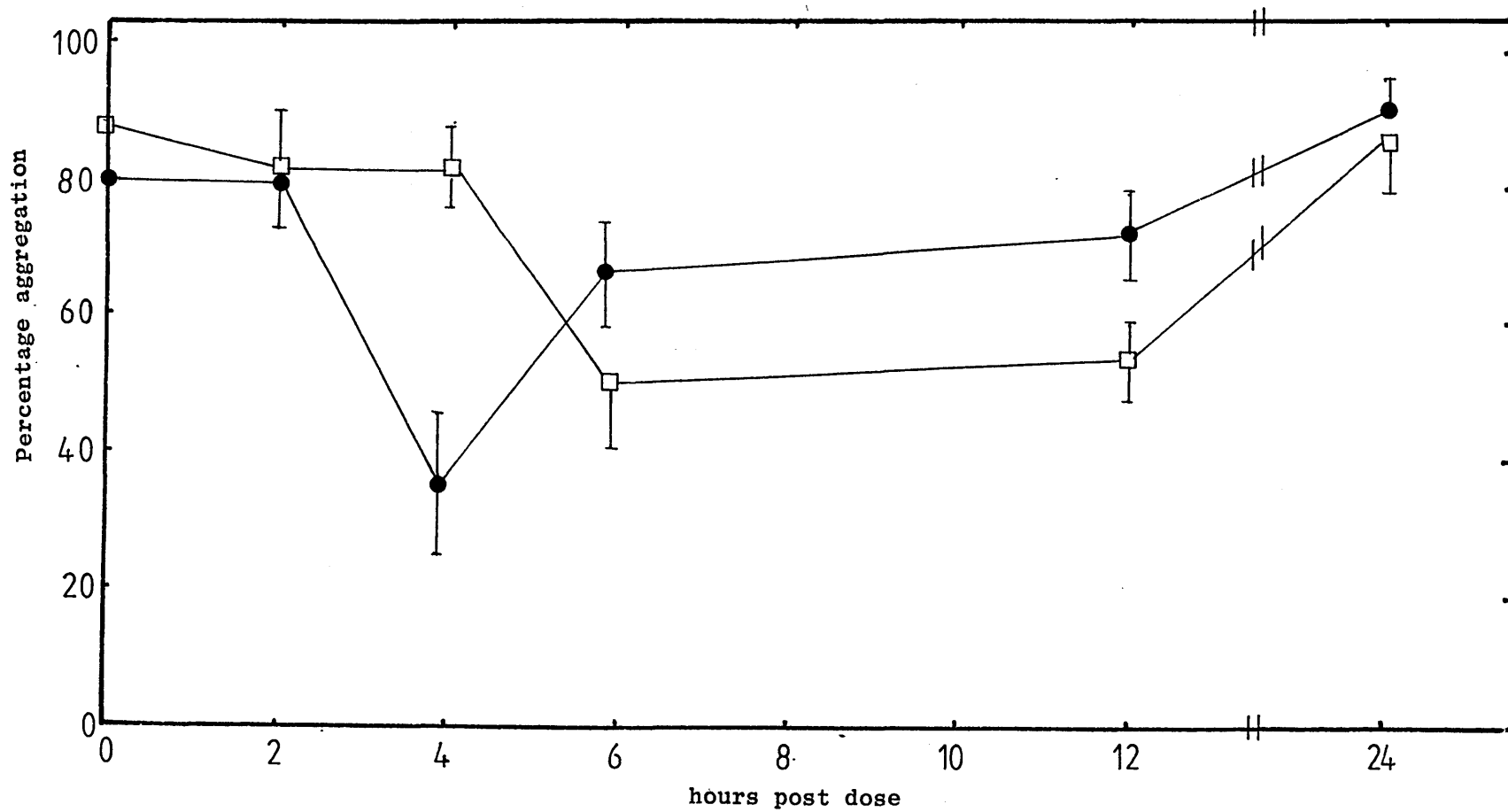
The effect of alkali-modified λ - and ι -carrageenan on ADP-induced platelet aggregation



Effect of alkali-modified λ -carrageenan (●) and ι -carrageenan (□) on platelet responsiveness to ADP (10 μ M). Control value was 72.5 ± 6.4 per cent when $n > 5$.

Graph B.4.2.4

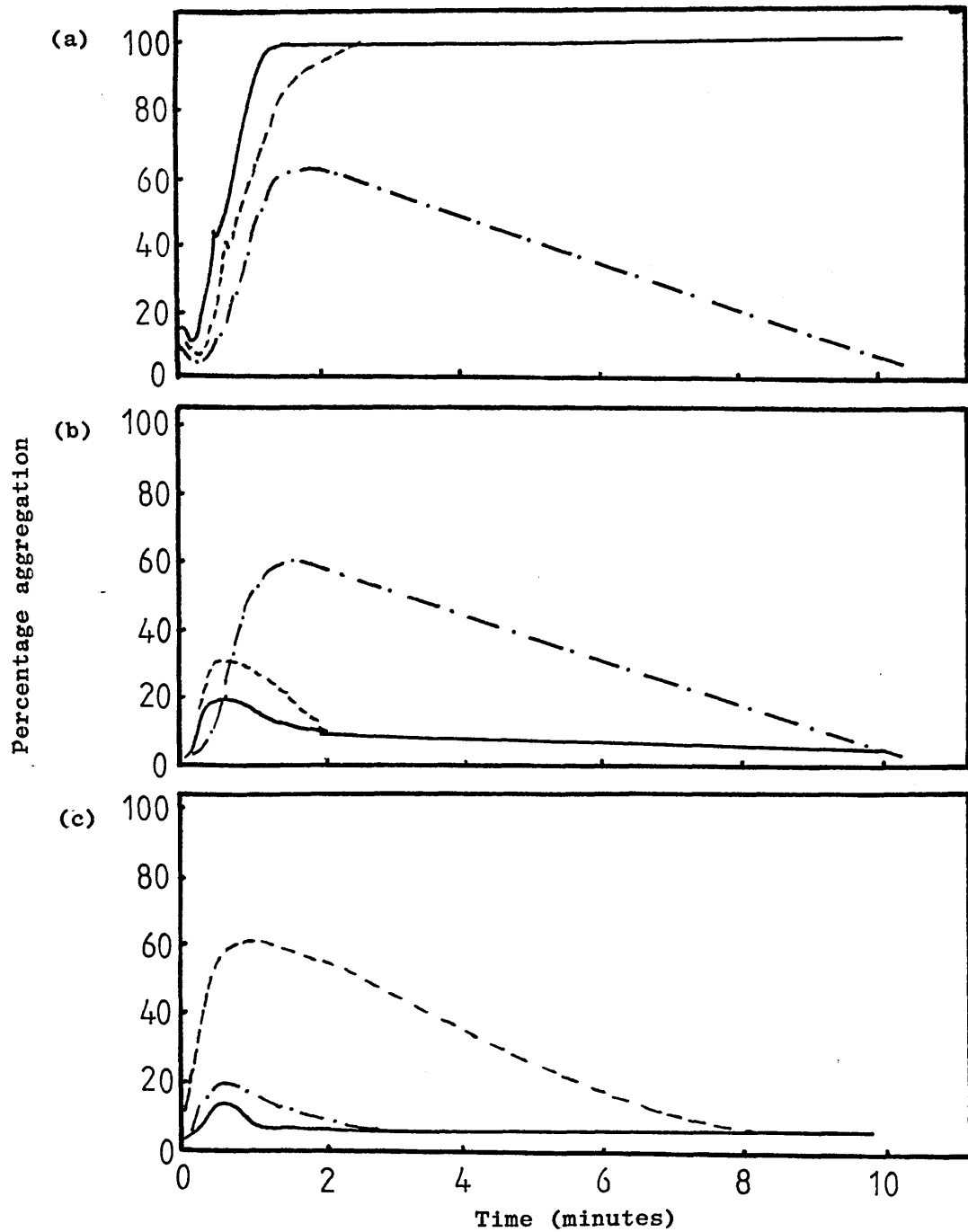
The effect of alkali-modified λ - and ι -carrageenan in vivo on ADP-induced platelet aggregation



Effect of alkali-modified λ -carrageenan (●) and ι -carrageenan (□) on platelet responsiveness to ADP (50 μ M). Control value was 82.5 ± 5.2 per cent when $n > 5$.

Graph B.4.3.1

Aggregation responses to thrombin at 4 hours post dose



Effect of carrageenan on thrombin induced aggregation at 4 hours
 (a) normal response (b) 1-carrageenan (c) alkali-modified λ-carrageenan.

— 1.5 NIH U ml⁻¹ thrombin
 --- 1.25 NIH U ml⁻¹ thrombin
 - · - 1.0 NIH U ml⁻¹ thrombin

Thrombin at $1.0 \text{ NIH U ml}^{-1}$ produced reversible aggregation in saline-treated animals resulting in 60 per cent aggregation. At 4 hours, alkali-modified λ -carrageenan reduced the thrombin response to 17 per cent, the response returning to normal level at 6 hours (Graph B.4.3.2, p. 130). Onset of ι -carrageenan antiplatelet effect occurred at 6-12 hours, with a normal response occurring at 24 hours.

A thrombin concentration of $1.25 \text{ NIH U ml}^{-1}$ resulted in a distinct first phase of aggregation, followed by second phase with thrombus formation. In both cases, the carrageenans exerted a potent anti-thrombotic effect in the 2-12 hour period (Graph B.4.3.3, p. 131). The inhibitory effect of ι -carrageenan was observed to be greater than that of alkali-modified λ -carrageenan, with a value as low as 26 per cent at 4 hours.

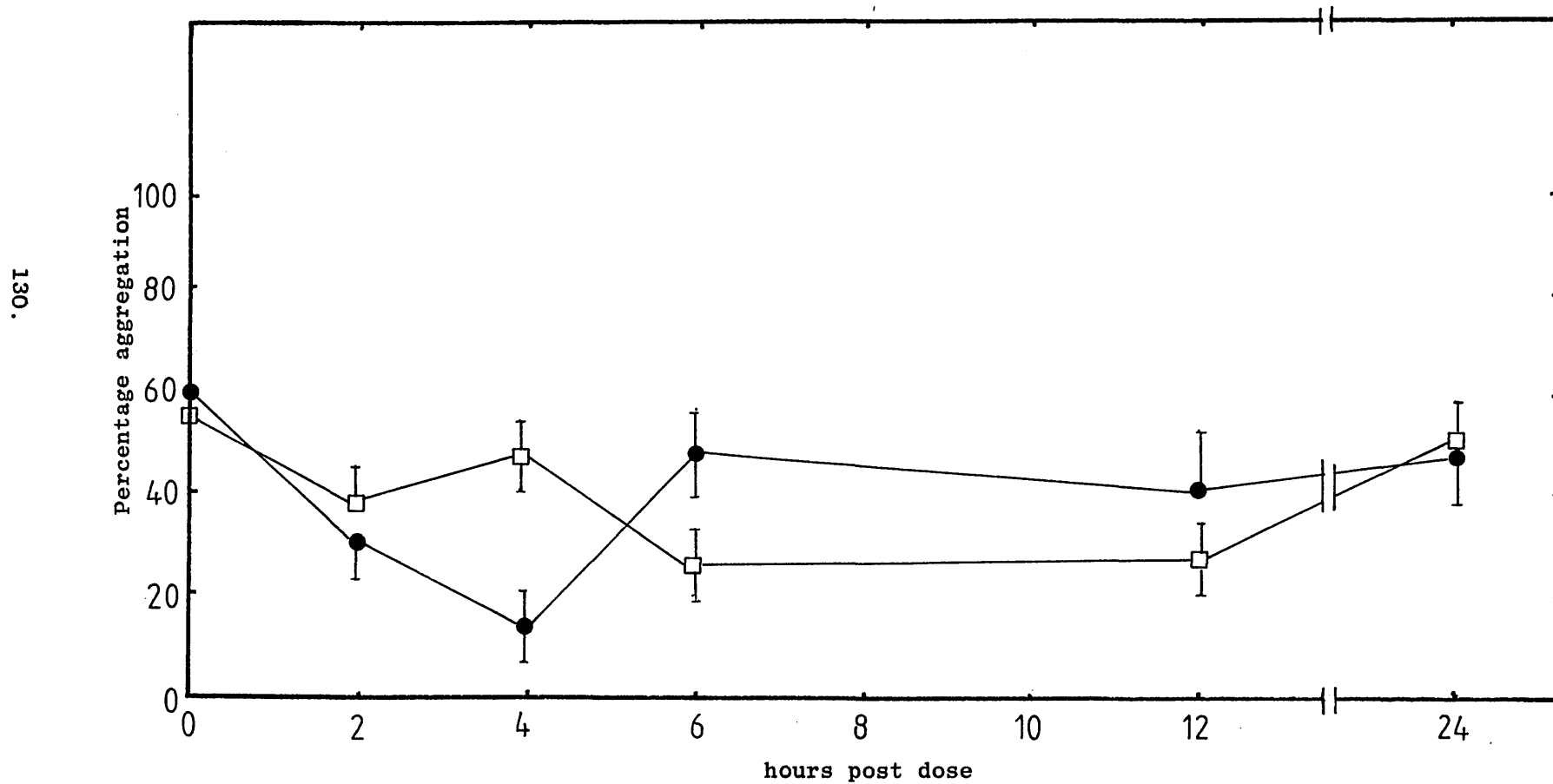
A thrombin concentration to $1.5 \text{ NIH U ml}^{-1}$ produced second phase irreversible aggregation in plasma from saline-treated animals. With alkali-modified λ -carrageenan, inhibition of thrombin activity was maximal at 6 hours (Graph B.4.3.4, p. 132). This effect was maintained over a 2-12 hour period, with only 8-11 per cent aggregation being observed. With ι -carrageenan, inhibition of thrombin activity was seen only between 4-6 hours.

B.4.4 Platelet adhesiveness studies

The assay for platelet adhesiveness was devised to gauge the influence of carrageenans on the induction of platelet "stickiness". Both carrageenans administered were shown to decrease platelet adhesiveness over an initial 2-4 hour period, with the ι -carrageenan effect extended to 12 hours (Graph B.4.4.1, p. 133).

Graph B.4.3.2

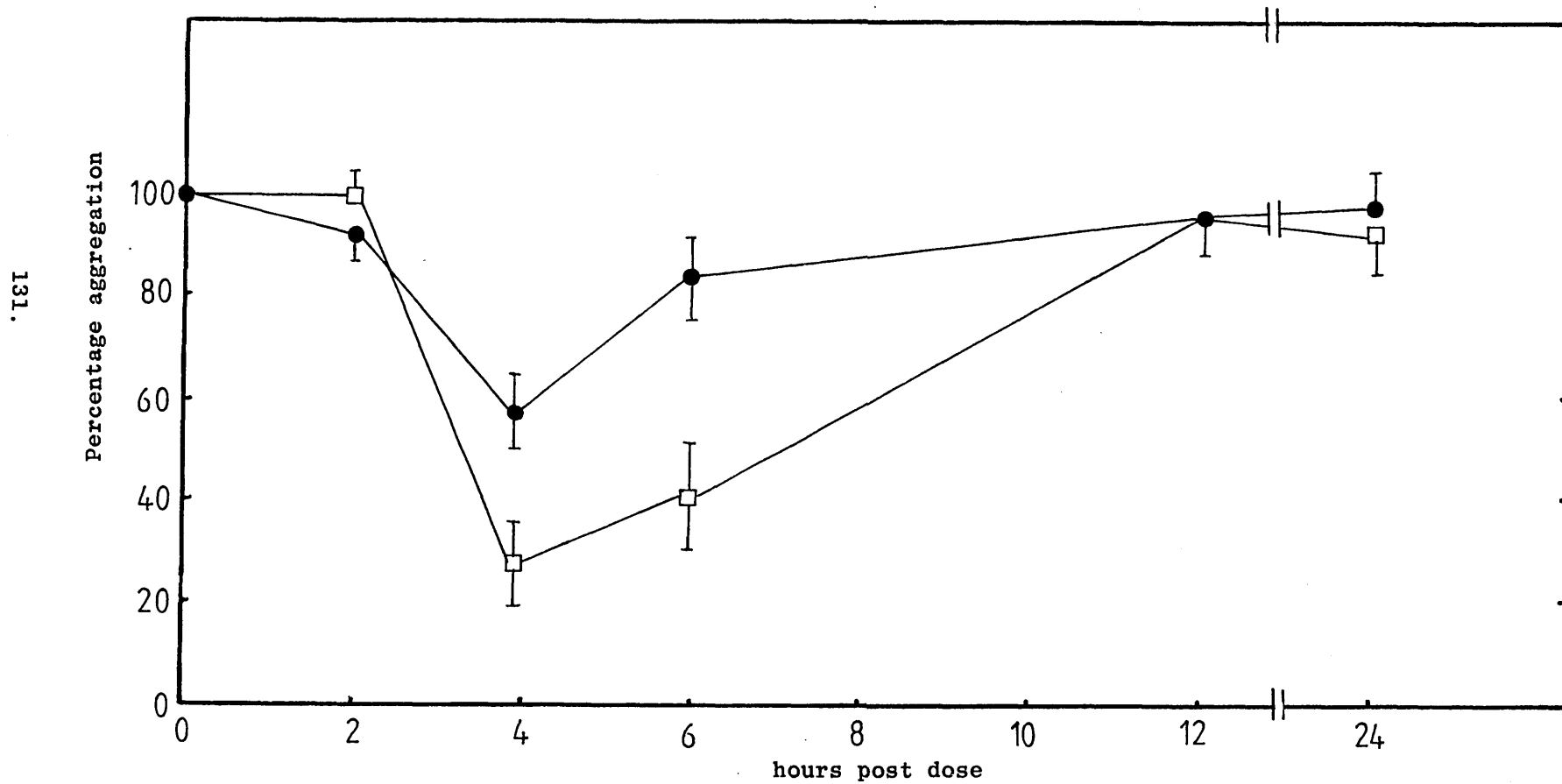
The effect of alkali-modified λ - and ι -carrageenan on thrombin-induced aggregation



Effect of alkali-modified λ -carrageenan (●) and ι -carrageenan (□) on platelet responsiveness to thrombin ($1.0 \text{ NIH U ml}^{-1}$). Control value was 58.0 ± 12.5 per cent when $n > 5$.

Graph B.4.3.3

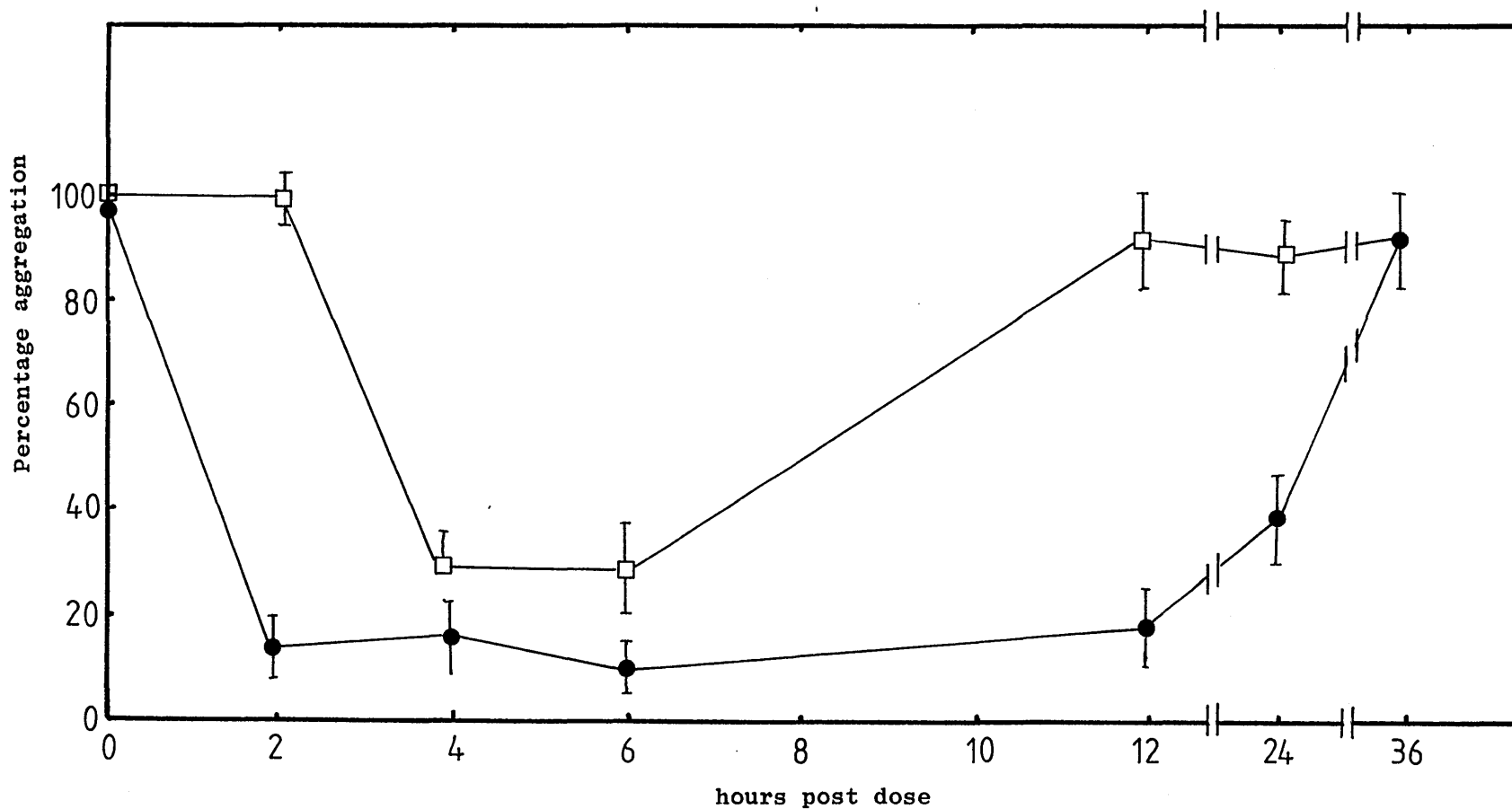
The effect of alkali-modified λ - and ι -carrageenan on thrombin-induced platelet aggregation



Effect of alkali-modified λ -carrageenan (●) and ι -carrageenan (□) on platelet responsiveness to thrombin ($1.25 \text{ NIH U ml}^{-1}$). Control value was 100 per cent when $n > 5$.

Graph B.4.3.4

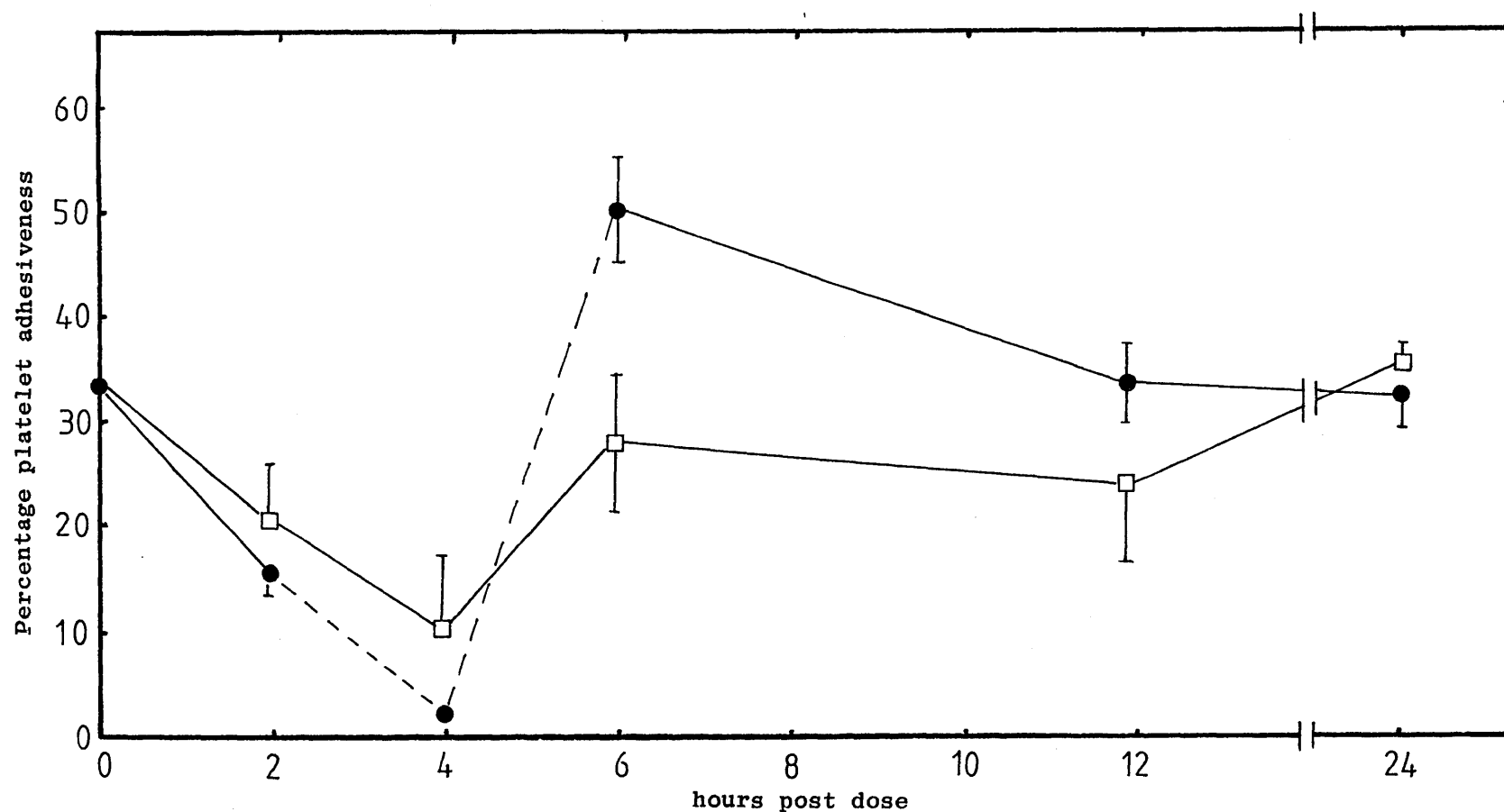
The effect of alkali-modified λ - and ι -carrageenan on thrombin-induced platelet aggregation



Effect of alkali-modified λ -carrageenan (●) and ι -carrageenan (□) on platelet responsiveness to thrombin ($1.5 \text{ NIH U ml}^{-1}$). Control value was 100 per cent when $n > 5$.

Graph B.4.4.1

The effect of alkali-modified λ - and ι -carrageenan on platelet adhesiveness



Effect of alkali-modified λ -carrageenan (●) and ι -carrageenan (□) on platelet adhesiveness following carrageenan administration. Control value was 34.8 ± 2.0 per cent when $n > 5$.

When platelets were observed microscopically over the 2 and 6 hour period they were found to be normal. In a few instances, platelets were seen to have agglutinated. At 4 hours, however, platelets obtained from alkali-modified λ -carrageenan-treated animals were found to have an irregular shape unlike either a normal or activated platelet. The cells were observed to be highly irregular in shape and difficult to count. The platelets had returned to normal in samples obtained from animals 6 hours post injection. In these alkali-modified λ -treated animals, there was an increase in platelet adhesiveness at 6 hours; however, platelets had returned to normal within a 12 hour period.

B.5 The immune response

B.5.1 Antigen concentration in serum

Maximum antibody response to sheep red blood cells (SRBC) injected i.p. was achieved at 4×10^{10} cells kg^{-1} (Graph B.5.1.1, p. 136). Comparison with literature resulted in a value of 2.5×10^{10} cells kg^{-1} being used to avoid tolerance induction (Thomson et al., 1981; Schneidkraut and Loegering, 1981).

B.5.2 Antibody response to 1-carrageenan administration

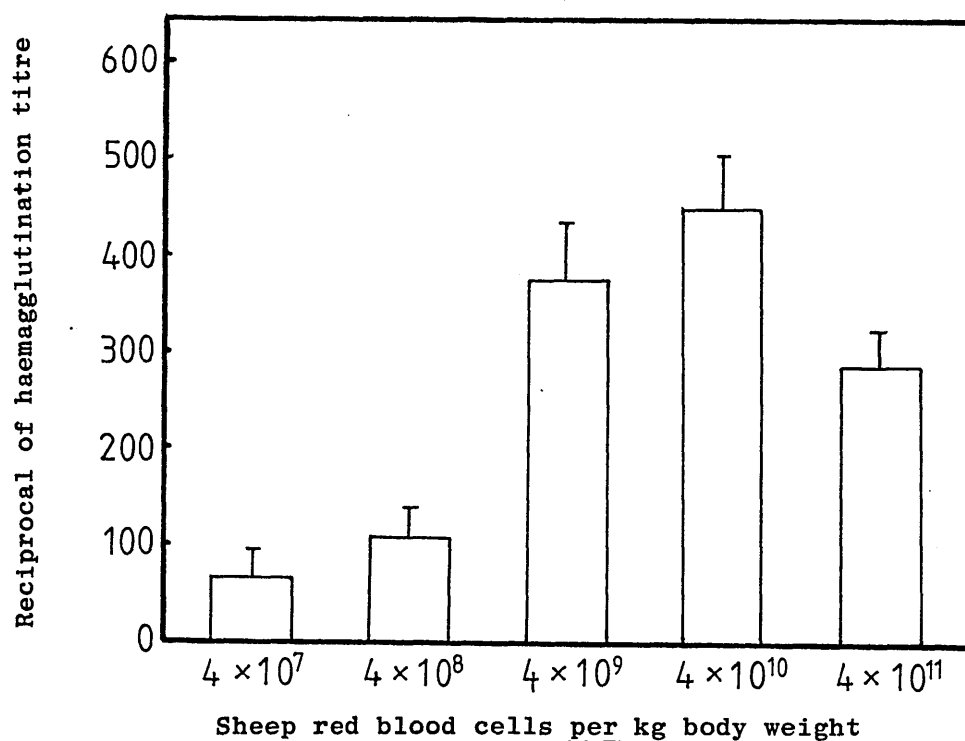
Results illustrated on Graph B.5.2.1 (p. 137) indicated that 1-carrageenan injected i.p. 24 hours before antigen (2.5×10^{10} cells kg^{-1} , i.p.) was immunosuppressive at all concentrations administered.

Post mortem investigation of all animals showed no immediately obvious pathological abnormalities except in animals receiving 40-60 mg kg^{-1} where several white plaques of 2-3 mm diameter were loosely adherent to the liver surface and similar material had converged and formed a large discrete sac round the caudate lobe of liver. Inflammation of the duodenum was evident in some of these animals.

Histological examination of the liver showed that the sac was surrounded by a continuous mesoendothelial sheath. There was no distinct organisation of cells with the sac, but large multinucleated macrophages containing carrageenan were observed attached to, or lying close to, areas of carrageenan. Some cellular debris due to lysis was evident as were actively proliferating fibroblasts and polymorphs. Similar observations were made with sections of the duodenum, the only difference being that the inflammatory response

Graph B.5.1.1

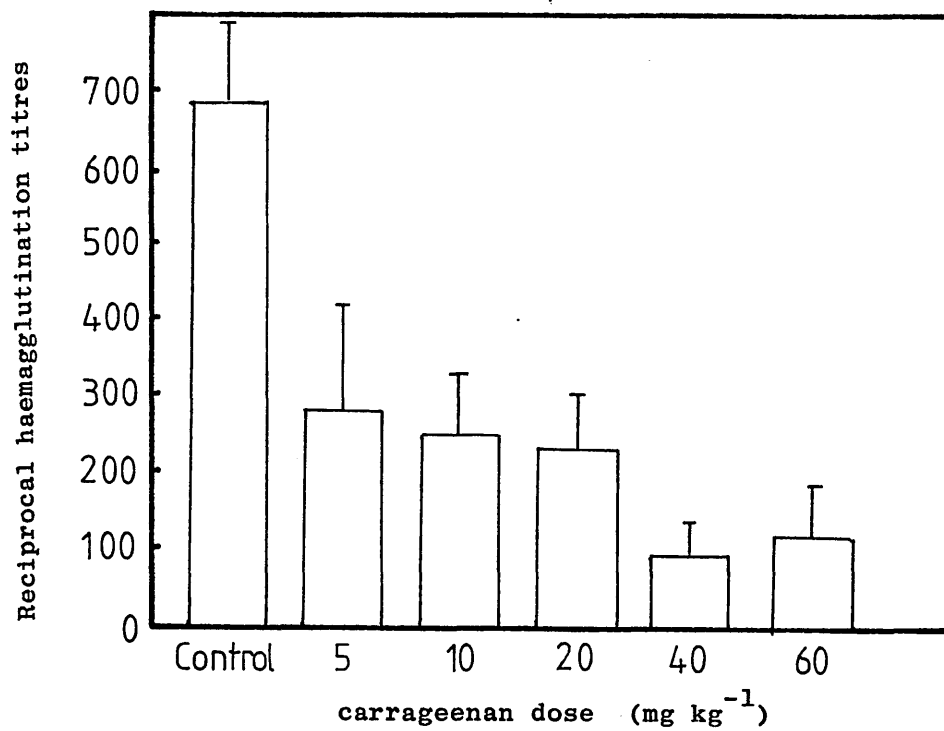
The effect of antigen on induction of the immune response



Effect of 1-carrageenan on antibody titres to concentrations of sheep red blood cells injected i.p. Each bar represents the mean + S.E. of 5 animals 8 days after antigen.

Graph B.5.2.1

The effect of λ -carrageenan on antibody
titres to sheep red blood cells



Effect of various concentrations of λ -carrageenan administered i.p. 24 hours prior to antigen (2.5×10^{10} SRBC kg⁻¹, i.p.) as assayed by the 8 day haemagglutination response. Each bar represents the mean + S.E. of 5 animals.

was present within the tissue and infiltrating blood vessels were seen to contain many inflammatory cells.

B.5.3 Total immunoglobulin levels

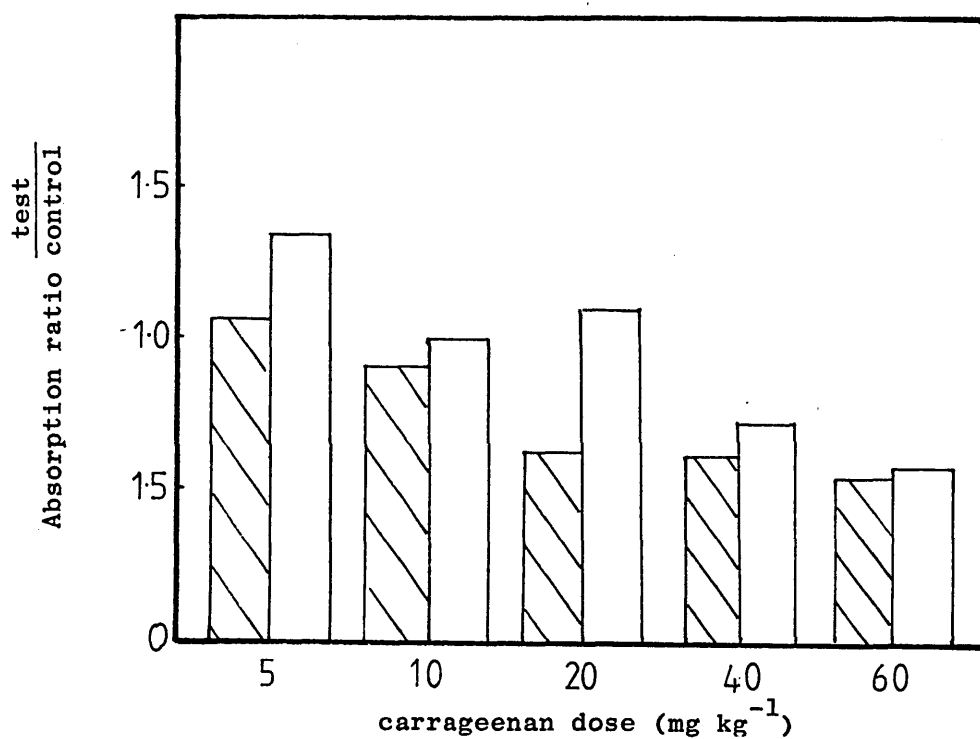
Serum from the previous experiment was assayed for total IgM and IgG levels by the enzyme-linked immunosorbent assay (ELISA). Results were obtained and expressed as ratios where a value less than 1.0 indicated suppression of the total IgM and IgG level. A trend similar to that of the haemagglutination assay was observed with total IgM and IgG levels (Graph B.5.3.1, p. 139).



B.5.4 The time of carrageenan injection in relation to antigen

Results expressed on Graph B.5.4.1 (p. 140) indicate that the time of injection of carrageenan in relation to antigen administration had no statistical significance when assayed by both the eight day haemagglutination assay and ELISA (Graphs B.5.4.1 and B.5.4.2, p. 140 and p. 141). The control level of this and subsequent repeat assays was found to be extremely low in comparison with previous experiments, thus the results obtained were invalid.

Graph B.5.3.1

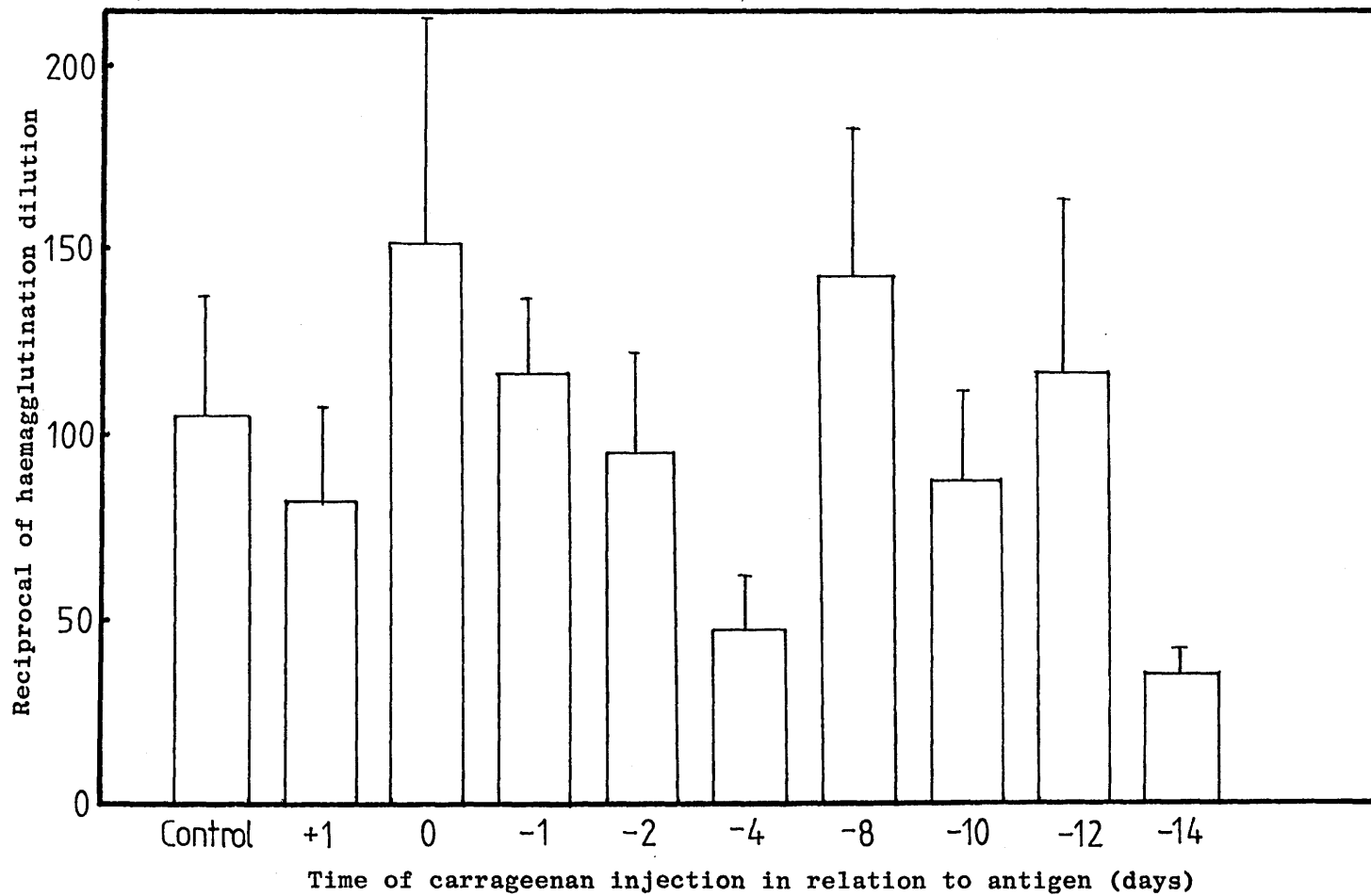
The effect of 1-carrageenan on total immunoglobulin levels



Effect of 1-carrageenan on total IgM  and IgG  levels as assayed by ELISA. Each histogram represents the mean of 5 animals per group.

Graph B.5.4.1

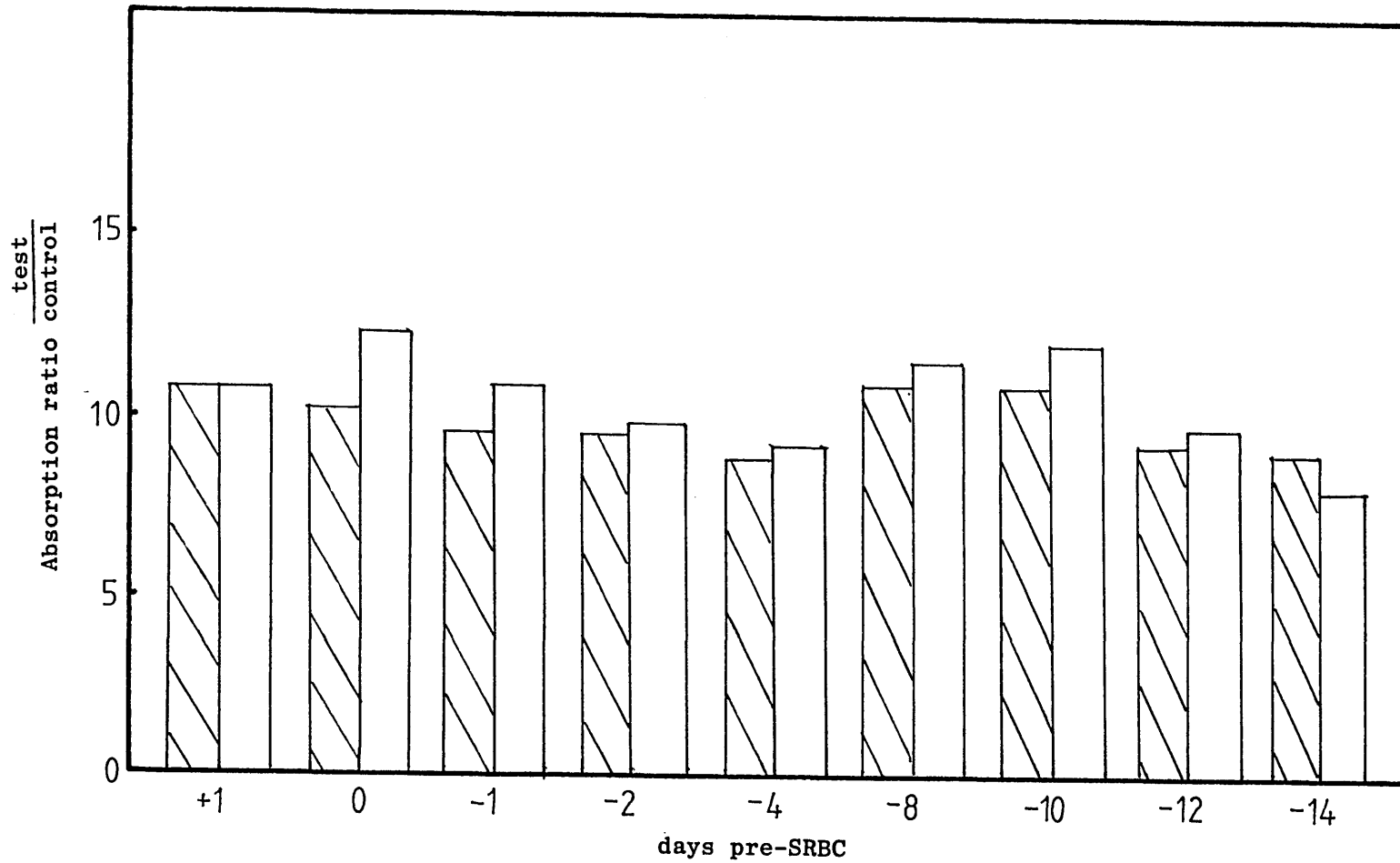
The effect of 1-carrageenan on antibody titres when injected in relation to antigen





Effect of 1-carrageenan (20 mg kg^{-1} i.p.) on the antibody titre when injected in relation to time of antigen (2.5×10^{10} SRBC kg^{-1}) as assayed by the 8 day haemagglutination assay.

Graph B.5.4.2

The effect of 1-carrageenan on IgM and IgG levels in response to sheep red blood cells



Effect of 1-carrageenan (20 mg kg^{-1}) on IgM  and IgG  levels in serum in response to SRBC ($2.5 \times 10^9 \text{ cells kg}^{-1}$) when administered pre-SRBC.

B. 6 Lipoprotein assay

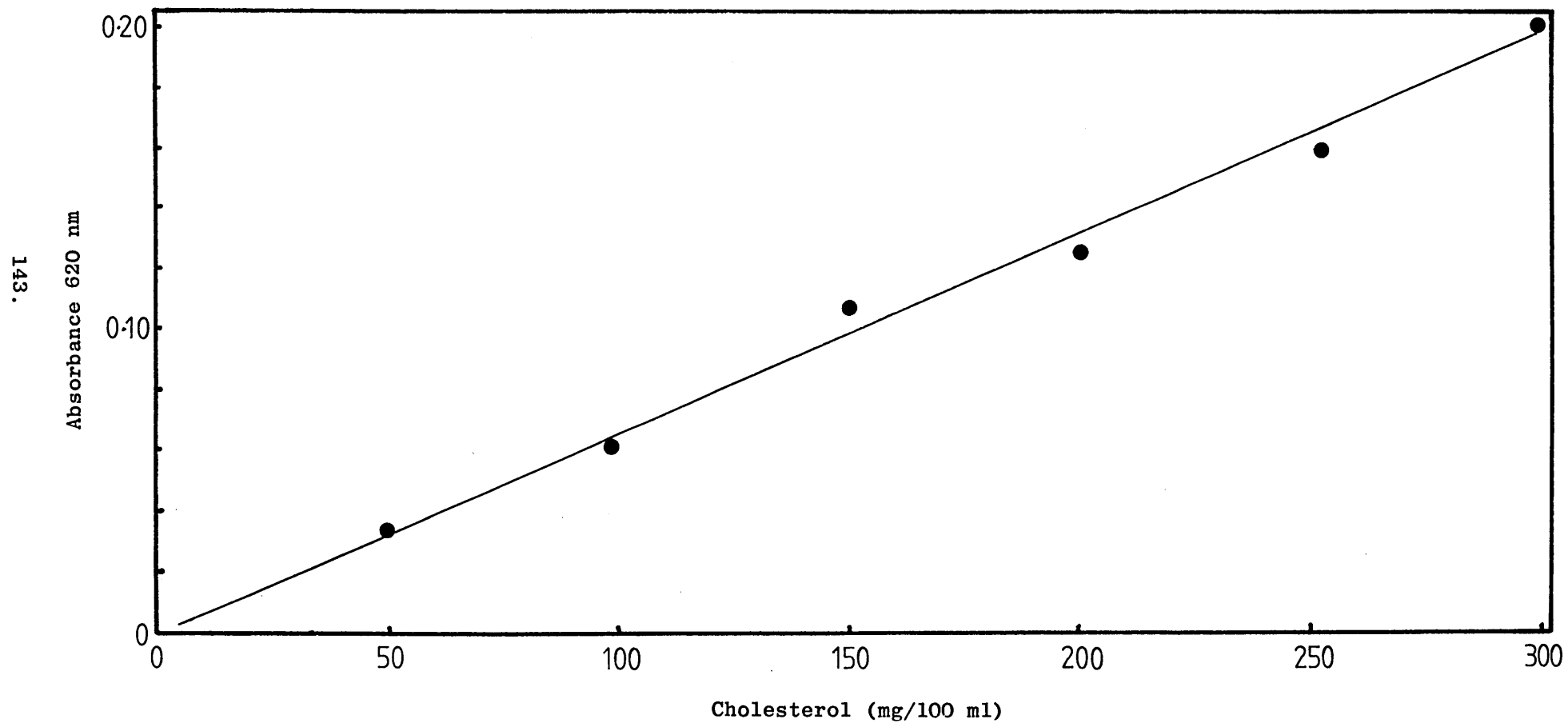
B.6.1 Serum lipoprotein studies

Four principal lipoprotein fractions, very low density lipoprotein (VLDL), intermediate density lipoprotein (IDL), low density lipoprotein (LDL) and high density lipoprotein (HDL) were separated by density ultracentrifugation and total cholesterol measured against a standard curve of cholesterol (Graph B.6.1a, p. 143). With alkali-modified λ -carrageenan-treated animals very high levels of VLDL and IDL were observed at 0-2 hours, with VLDL again maximal over the 6-12 hour period. Elevation of LDL occurred at 2 hours and 12 hours but no significant change was observed with HDL levels (Graphs B.6.1.1 - B.6.1.4, p. 143-146).

The serum from λ -carrageenan-treated animals indicated that this carrageenan had no effect on VLDL levels but increased IDL, LDL and HDL levels at 6 hours. The levels returned to normal at 24 hours (Graphs B.6.1.1 - B.6.1.4, p. 143-146)

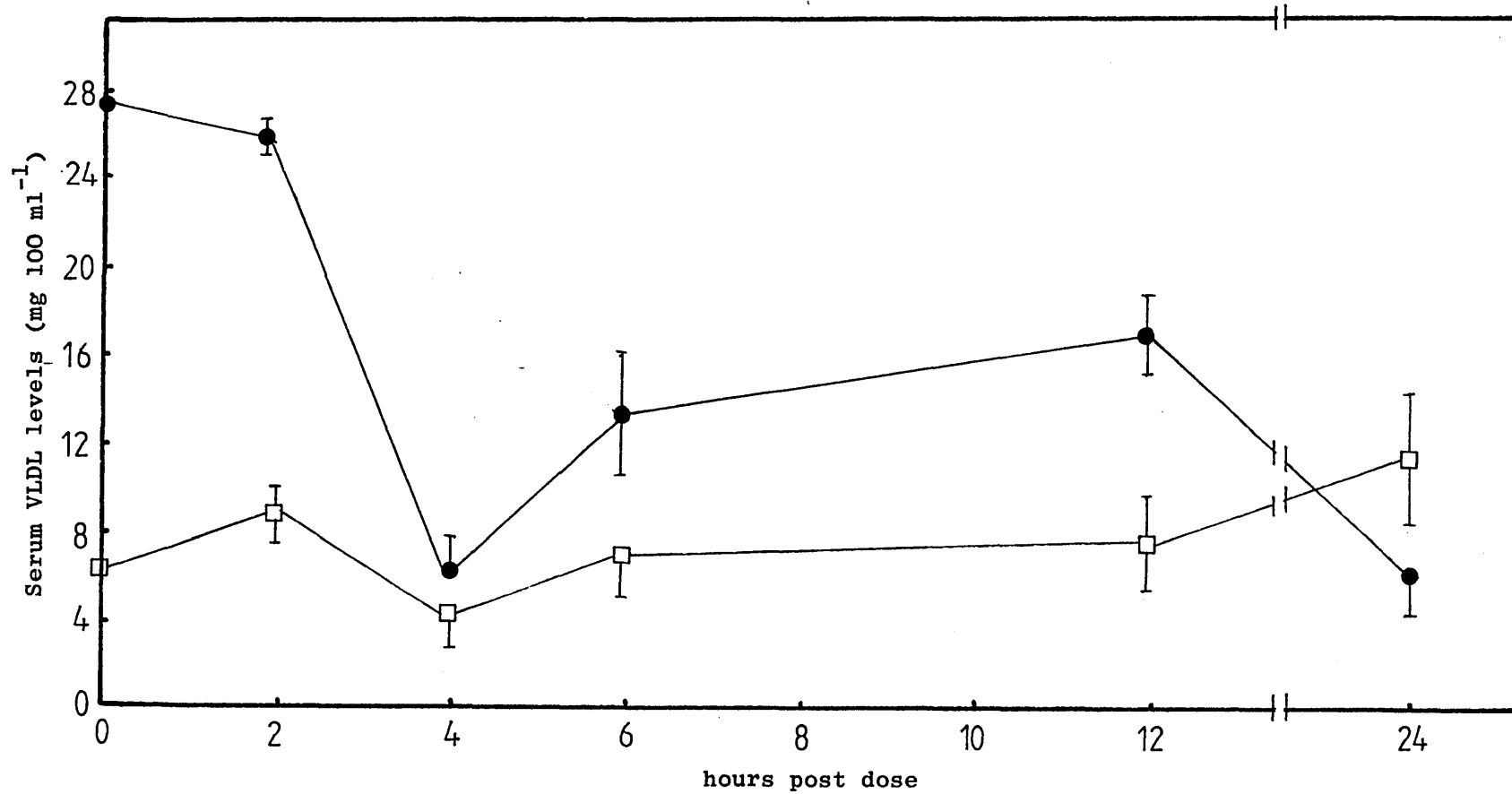
Graph B.6.1a

Standard curve of cholesterol for lipoprotein assays



Graph B.6.1.1

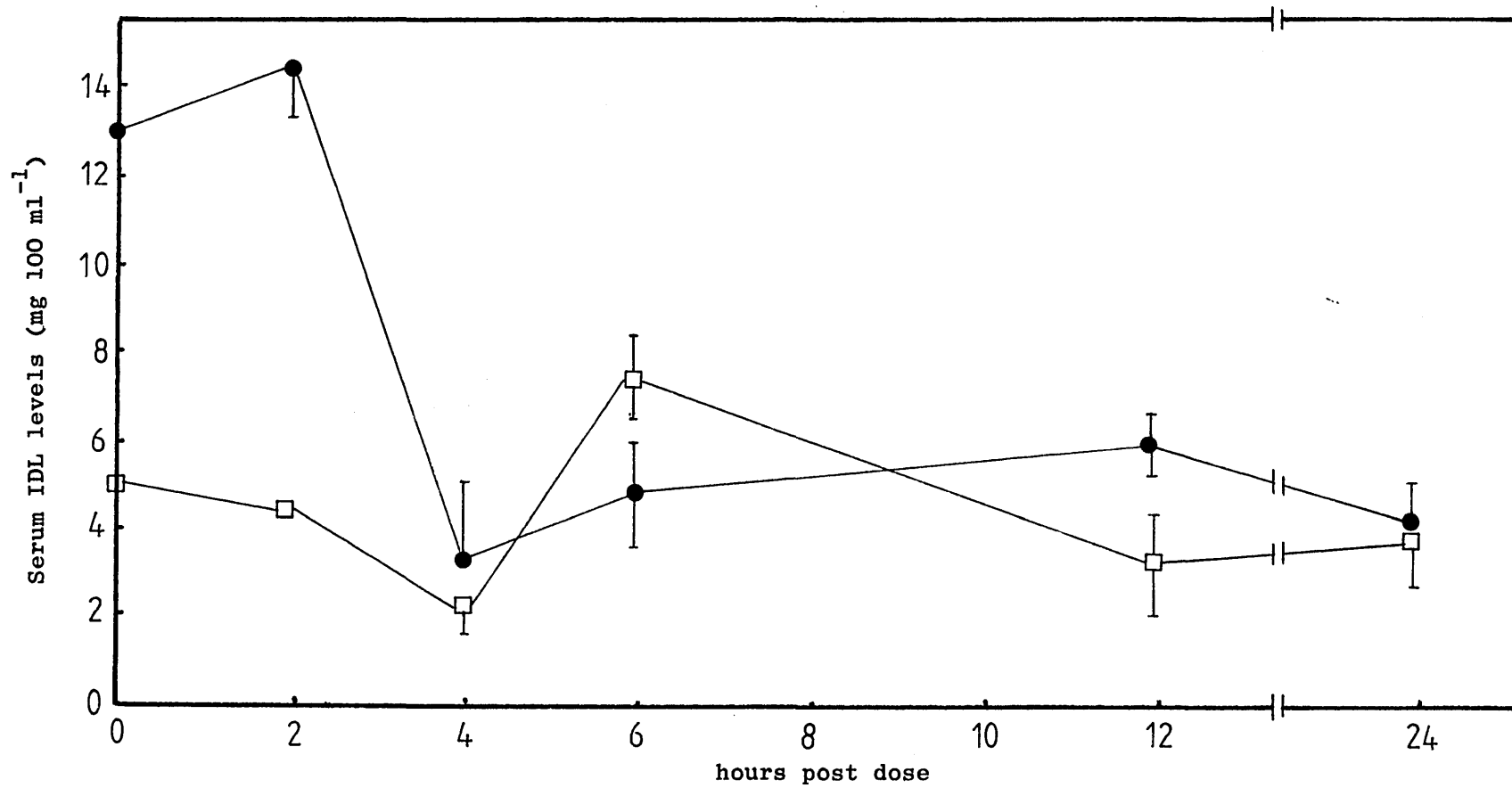
The effect of alkali-modified λ - and ι -carrageenan on serum very low density lipoprotein levels



Effect of alkali-modified λ -carrageenan (●) and ι -carrageenan (□) on serum VLDL levels.

Graph B.6.1.2

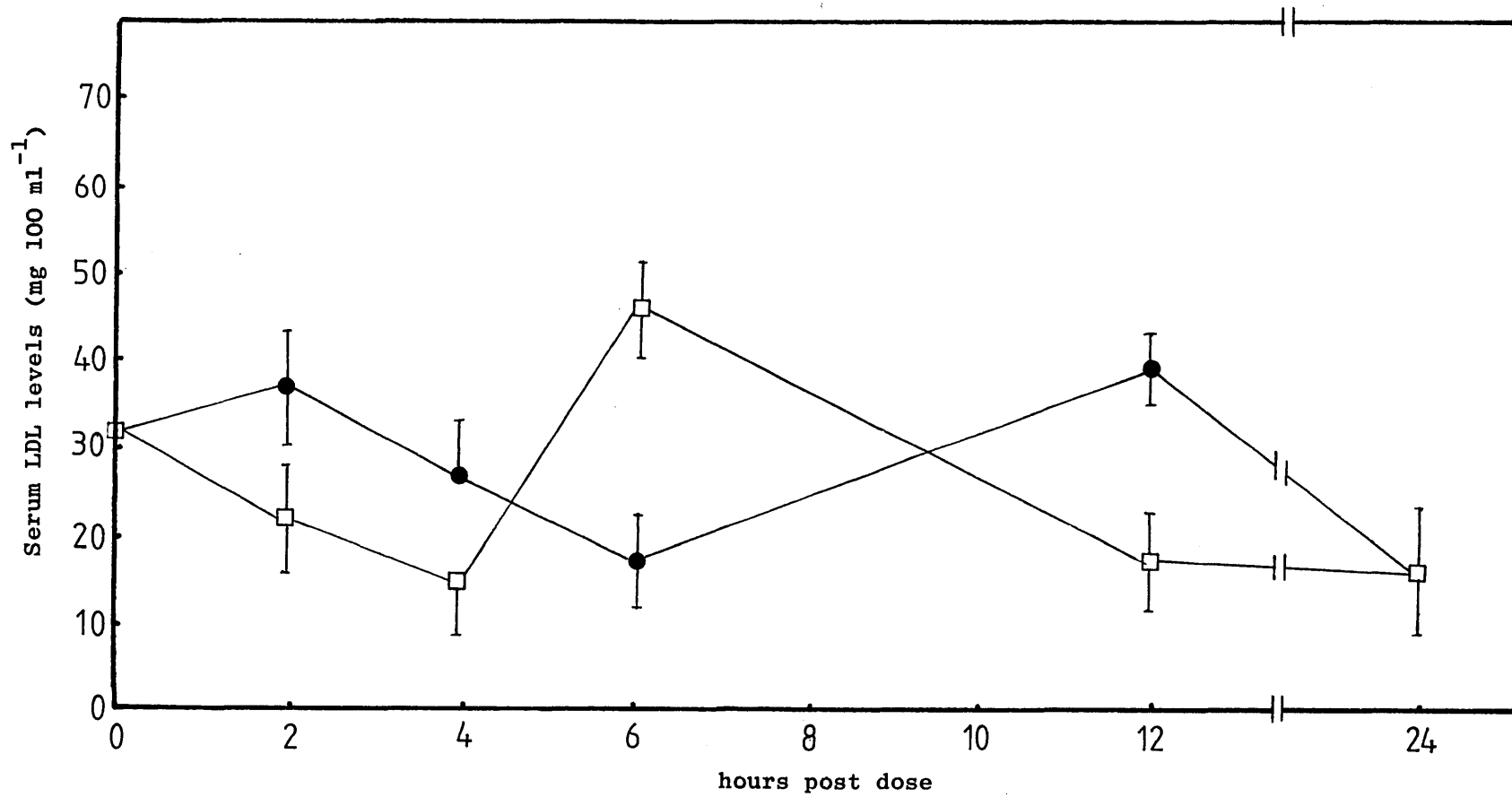
The effect of alkali-modified λ - and ι -carrageenan on serum intermediate density lipoprotein levels



Effect of alkali-modified λ -carrageenan (●) and ι -carrageenan (□) on serum IDL levels.

Graph B.6.1.3

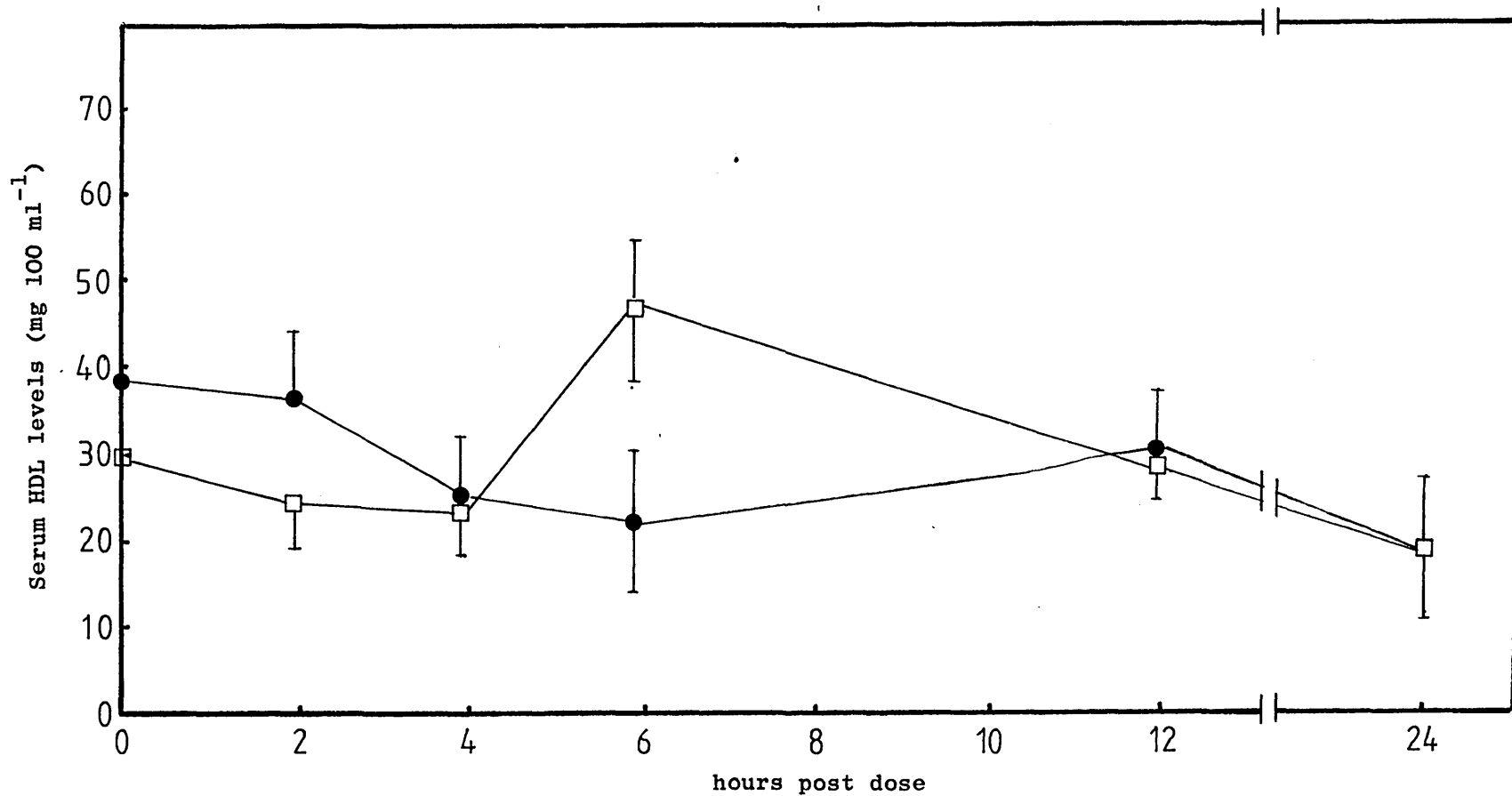
The effect of λ - and ι -carrageenan on serum low density lipoprotein levels



Effect of alkali-modified λ -carrageenan (●) and ι -carrageenan (□) on serum LDL levels.

Graph B.6.1.4

The effect of alkali-modified λ - and ι -carrageenan on serum high density lipoprotein levels



Effect of alkali-modified λ -carrageenan (●) and ι -carrageenan (□) on serum HDL levels.

V DISCUSSION

1. Blood clotting

Carrageenans were used in the present study to investigate their effects on the pathways and cell systems of blood coagulation centering on Factor XII activation. By selection of appropriate in vitro assays, carrageenans were shown to have effects primarily as anticoagulants (Graphs A.1.1.1 - A.1.8.1, p. 78 and 87). Extension of the studies to examine carrageenan effects following administration to rats showed the alkali-modified λ -carrageenan and λ_{Na} -carrageenan to possess the most anticoagulant activity of the fractions tested (Results, p. 107).

With respect to carrageenan effects, route of administration will influence absorption and distribution of the polysaccharides within the body fluids. Not least of all is the interaction of these polyelectrolytes with both humoral and cellular elements within the peritoneal cavity. The potential interactions which could take place are as follows: Carrageenan, as an anionic electrolyte, would be expected to associate directly with positively-charged plasma proteins yielding a macroionic complex. Proteins are amphoteric polyelectrolytes and depending on their isoelectric points may be more negatively or positively-charged at physiological pH. Direct interaction of the proteins which are positively-charged with the negatively-charged carrageenan could therefore occur (Comper and Laurent, 1978).

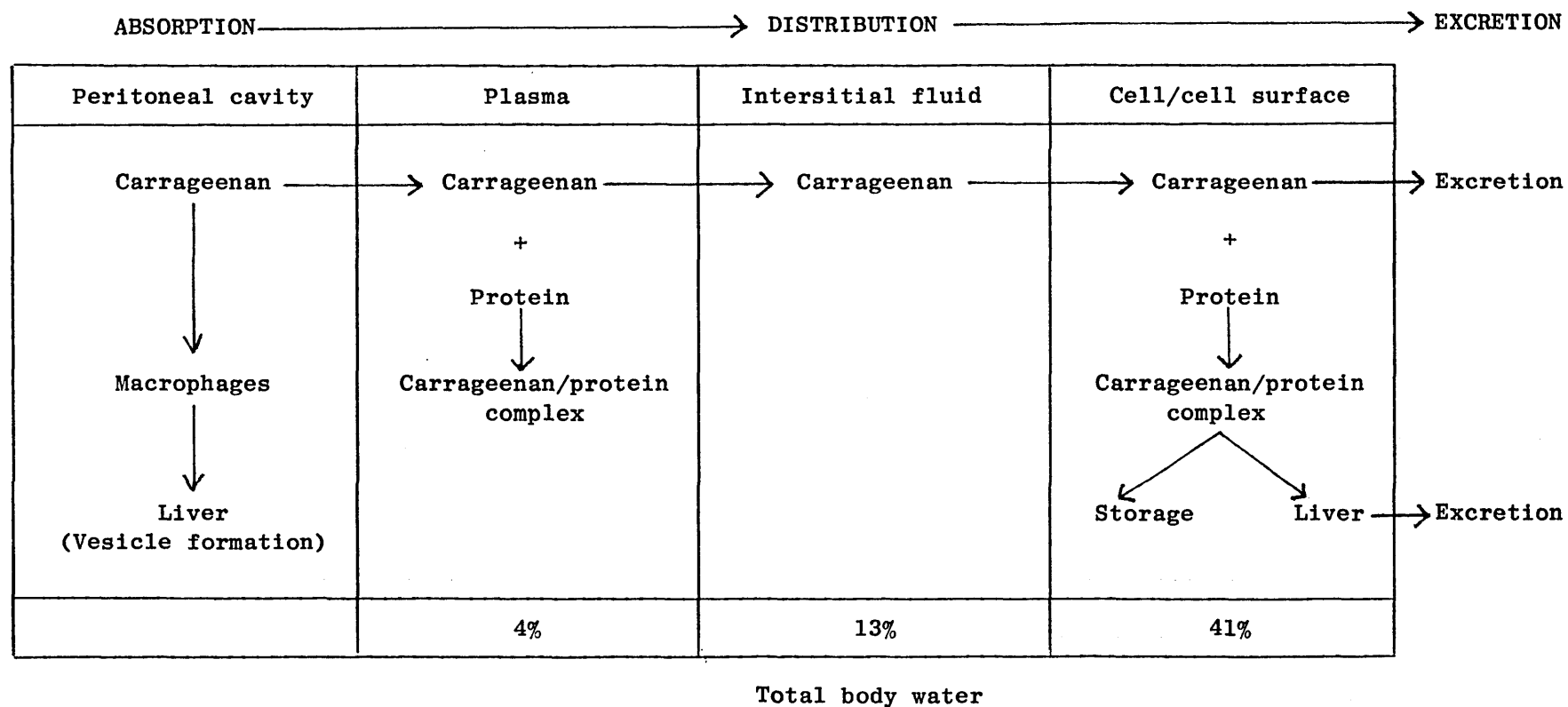
The occurrence of the anionic groups at frequent intervals along the polyelectrolyte chain results in the sharing of charge. Hence the carrageenan has a shell of negative charges of greater or lesser

density along its length, rather than individual point charges. The overall conformation of the carrageenan may also be influenced by the particular salt formation which could considerably influence potential interactions with plasma proteins, for example, the calcium salt of the carrageenan where the calcium ion forms a bridge between negatively-charged proteins and the negatively-charged carrageenan. Alkali-modified λ -carrageenan, largely a linear conformation, may act in a similar manner to any negatively-charged surface such as glass or kaolin, and may form a template for protein alignment. λ -Carrageenans in some studies may adopt a helical structure in which chains are held by electrostatic and hydrogen bonding (Rees, 1961). This structure forms a more viscous gel matrix compared to the alkali-modified λ -carrageenan. The κ -carrageenans form yet more gel networks, again by interchain bondings (Chakraborty and Hansen, 1971); however, proteins may bind at the interconnecting strands between helices resulting in the stabilisation of the protein (Figure B.1, p. 150).

With a negatively-charged galactan sulphate and a positively-charged protein moiety, electrostatic interactions take place between the sulphate half-esters of the galactose unit and the protonated N-terminal amino group on the protein. Alignment may not be total, and intermediate or transitional degrees of association may occur due both to electrical interaction and structural compatibility.

When examined in functional blood clotting assays, heparin and carrageenans, with the exception of κ -carrageenan, were anticoagulant in vitro (Graphs A.1.1.1 to A.1.8.1, p. 78 to 87). Other authors observed similar responses (Ratnoff, 1954; Hawkins and Leonard, 1963; Anderson and Duncan, 1965; Kindness, Long and Williamson, 1980a).

Figure B.1



When carrageenan was injected into the peritoneum, maximal anticoagulant effects were found with the alkali-modified λ -carrageenan and the γ_{Na} -carrageenan; therefore, studies ex vivo were centered on these two carrageenans in an attempt to investigate the carrageenan influence on the blood coagulation pathways.

The in vitro studies showed that the carrageenans activated Factor XII but that this effect was obscured by the anticoagulant effect exerted by the same compounds (Graph A.1.2.1, p. 79). Because heparin, a mammalian sulphated polysaccharide of superficially similar structure to carrageenan, is a well-known anticoagulant, effects of carrageenan and heparin on blood clotting were compared. It was initially suggested by Rosenberg and Damus (1973) that heparin affected the early stages of the intrinsic pathway of coagulation by interacting with the proteinase inhibitor antithrombin III (AT III) which accelerates the neutralisation of Factors XII, IX, X, XI and thrombin. Heparin also interacts directly with thrombin (Kindness, Long and Williamson, 1980a) although Gitel (1978) showed that the binding of thrombin to heparin was less strong than that of thrombin to AT III.

Within this study, in vitro assays showed that removal of AT III from plasma reduced the carrageenan influence on thrombin (Graph A.1.7.1, p. 86) but had little effect on the carrageenan influence on Factor X (Xa) (Graph A.1.6.1, p. 85). Hence, carrageenans could act in a heparin-like manner via complex formation with AT III but also inhibit anticoagulant activity independent of AT III as shown in studies employing AT III-depleted plasma (Graph A.1.8.1, p. 87)

Carrageenans studied ex vivo showed anticoagulant effects when assayed by thrombin clotting time but only the alkali-modified λ -

carrageenan was anticoagulant at a Factor XII and Factor X (Xa) level (Graphs B.1.1.1 to B.1.3.1, p.109 to 111). These results, in conjunction with the in vitro results, may be explained in the following way:-

The carrageenans could act in a structure-dependant manner through complex formation with suitable plasma proteins and as HMWK and fibrinogen via appropriate binding groups. The alkali-modified λ -carrageenan may essentially be a straight-chained molecule which may form a suitable matrix on which serine proteinases and/or their substrates or inhibitors may align.

The ι - and κ -carrageenans form viscous gel matrices and, although highly negatively-charged on the surface of the matrix, the internal negative charges may be masked. Hence, interaction with proteinases or their inhibitors may be reduced compared with the alkali-modified λ -carrageenan.

The activity of the carrageenans as electrolytes should also be considered; alignment of the positively-charged proteins along a negatively-charged particle would be more efficient the greater the charge density. It would therefore follow that the alkali-modified λ -carrageenan would be more anticoagulant than either the ι - or κ -carrageenans. However, in a large number of the assays within this thesis, the alkali-modified λ -carrageenan was not the most active anticoagulant so charge density may not be the only contributing factor.

Studies have been carried out on heparin-protein interactions (Blackwell, Schodt and Gelman, 1977). These authors found that heparin caused conformational changes within the protein, primarily at the active site where high charge density occurs. This conformational change induced by heparin was found to alter the biological activity of the protein.

Studies with AT III, showed that heparin increases the activity of AT III towards thrombin neutralisation (Abildgaard, 1969). It was found by Hopwood et al. (1976) that segments of certain heparin chains contained AT III-binding sites of high affinity. How often these sites occurred per heparin chain could be determined by length and selection of the required disaccharide units. This concept that heterogeneous regions along the chain are important, in determining or controlling binding with selected plasma proteins and hence the biological activity of the galactan sulphates, emerges from these studies (Hopwood et al., 1976). Also, affinity for a given complex will not only depend on the strength of the electrostatic interaction but also on the closeness of fit of the two molecules (Jacques, 1979).

These hypotheses may be applied to the results of the amidolytic assays carried out in vitro and ex vivo for Factor X (Xa) and thrombin. The carrageenans were found not to directly affect the rate of Factor X (Xa) amidolysis in vitro (Graph A.1.9.1, p. 88), but ex vivo slight inhibition occurred at 12 hours post carrageenan administration with both the carrageenan fractions studied (Graph B.1.4.1, p. 113). Thrombin amidolysis was inhibited by all concentrations of heparin but by only high carrageenan concentrations in vitro (Graphs A.1.10.2 to A.1.10.4, p. 91 to 93). This type of assay again highlights the difference between a functional clotting assay which takes into account factors other than that being directly examined and one which is more specific, such as the amidolytic assay, where only the direct activation of a serine proteinase is being studied.

Putative effects of the carrageenans on the intrinsic pathway of coagulation may be summarised by stating that there is a carrageenan interaction with Factor XII, Factor X and thrombin. The interaction

may be dependent on AT III-neutralisation of thrombin but no AT III involvement was observed with Factor X. This effect may prevent cleavage of fibrinogen to fibrin, resulting in an anticoagulant effect along with a reduction in FDP production and concomitant reduction in the response to an antigen. In ex vivo assays, alkali-modified λ -carrageenan was more anticoagulant than the λ_{Na} -carrageenan fraction, possibly due to the alkali-modified λ -carrageenan being of a higher charge density and therefore a more suitable electrolyte. The λ -carrageenan was found to act primarily in the neutralisation of the proteinase thrombin and correlation with in vitro results suggests that AT III may bind to specific areas of the molecule resulting in the increased activity of the antiprotease towards thrombin.

This thesis is based on the pathways associated with Factor XII activation and so the study was continued to ascertain the effects of carrageenan on fibrinolysis.

2. Fibrinolysis

Spontaneous generation of fibrinolytic activity in vitro or ex vivo could not be demonstrated in normal rat plasma. Only by acidification of the plasma, with the subsequent formation of a euglobulin precipitate, was it possible to show carrageenan activation of the fibrinolytic pathway. Euglobulin precipitation per se removes the vast majority of the inhibitors of fibrinolytic activity; however, proteins such as $\text{C}\bar{\text{I}}$ inhibitor may precipitate with the euglobulin fraction, resulting in Factor XIIa neutralisation by its complex formation with the inhibitor (Kluft, Brakman and Veldhuyzen-Stalk, 1976).

In the in vitro studies, addition of carrageenan to plasma before euglobulin precipitation resulted in an increase in fibrinolytic activity which was shown to be dependent on the concentration of carrageenan used. Maximal activity was found at 0.1 mg ml^{-1} with the κ -carrageenan (Graph A.2.1.1, p. 95). This type of response to carrageenan was also found in human plasma (Kindness, 1979) with the higher concentration of test galactan, the reduced fibrinolytic response may have been due to two effects. As the euglobulin fraction was formed, the ionic polymers may have co-precipitated with fibrinolytic inhibitors such as $\text{C}\bar{\text{I}}$ inhibitor and histidine-rich glycoprotein (Lijnen, Rylatt and Collen, 1983). Similarly, at the optimum test concentration for fibrinolysis, sequestration of free inhibitor by the polyanions could allow cleavage of plasminogen in an inhibitor-free environment.

The pH required to obtain maximal control fibrinolysis was found in this thesis to be lower for rat than for human plasma (Kindness, 1970). The method of euglobulin precipitation using low pH may not

be ideal, since additional plasma proteins could be required for fibrinolysis and could remain in the supernatant following euglobulin precipitation. Similarly, during addification of plasma the charge effect on the protein may have been altered resulting in the potential activator being precipitated or neutralised during complex formation. This view may be supported by the finding that fibrinolysis, ex vivo, was found to be inhibited by carrageenan (Graph B.2.1.1, p.115). At 2 hours post dose 1-carrageenan showed maximal inhibition while the alkali-modified λ -carrageenan effect ranged over the 2-6 hour period with optimal inhibition at 6 hours.

In an attempt to determine the sites at which carrageenan might be affecting the fibrinolytic pathway, ϵ -ACA, an inhibitor of plasminogen, was incorporated into plasma samples prior to euglobulin precipitation. The ϵ -ACA binds to plasminogen and inhibits its activation. Results expressed on Graph A.2.2.1, (p. 96) for in vitro studies showed that ϵ -ACA did decrease fibrinolytic activity. The carrageenan effect was reduced except in the case of the κ -carrageenan fractions where fibrinolytic activity increased two-fold over that detected in normal plasma.

When ϵ -ACA was incorporated into ex vivo plasma samples a decrease in the fibrinolytic inhibitory response resulted (Graph B.2.3.1, p.118). These results illustrate that the κ -carrageenan in vitro may inhibit the binding of ϵ -ACA to the plasminogen, possibly through a mechanism in which plasminogen is trapped in the interconnecting stands of the galactan sulphate gel matrix. This type of interaction may not occur with the other carrageenan structures. Alternatively, the κ -carrageenan may form the closest fit to the active site of the plasminogen, rendering it less susceptible to inhibitors in the plasma. Euglobulin

precipitation would then remove the inhibitor into the supernatant, allowing action of the proteinase on the fibrin plate.

Flufenamic acid inhibits $\text{C}\bar{\text{I}}$ inhibitor activity, therefore increasing the fibrinolytic activity since $\text{C}\bar{\text{I}}$ inhibitor inhibits plasmin action on fibrin (Donaldson and Harrison, 1982). In vitro, flufenamic acid increased only the κ -carrageenan induced fibrinolytic activity whilst ex vivo samples from alkali-modified λ -carrageenan treated plasma euglobulin showed decreased fibrinolysis at 4-6 hours post dose. These results suggest that $\text{C}\bar{\text{I}}$ inhibitor plays an important part in the regulation of fibrinolysis both in vitro and ex vivo. The κ -carrageenan, as with ϵ -ACA, may bind competitively to the $\text{C}\bar{\text{I}}$ inhibitor binding site thus initiating an increase in fibrinolysis.

The carrageenan in vitro could activate Factor XII and hence trigger plasminogen proactivator and prekallikrein along with the coagulation cascade (Kluft, 1978b). Carrageenan may cause precipitation of proteins such as $\text{C}\bar{\text{I}}$ inhibitor into the euglobulin precipitate and on resolubilisation of the precipitate, the inhibitor would be released into the supernatant to bind to plasminogen.

Alternatively, studies have been carried out with κ -carrageenan that illustrate how this polymer binds and protects proteins (Chakraborty and Hansen, 1971). The κ -carrageenan strands gel to form bundles of helices with no ability to react with protein. The loose ends of the helices serve as junction zones to connect other strands which form a three dimensional network and may trap protein molecules. In the euglobulin precipitation the κ -carrageenan may take up plasminogen activator at its junction zones and release the proteins into the supernatant on resolubilisation. Since the proteins have been protected

from the external environment no denaturation occurs. This could explain why κ -carrageenans are so highly fibrinolytic in vitro.

The in vitro studies showed that the carrageenan fibrinolytic response was concentration-dependent and this may explain the response observed ex vivo. Insufficient carrageenan may have been present in the circulation to induce a fibrinolytic response. Also, the carrageenan in vivo may cause changes in the precipitation pattern of the proteins and in the amount of plasminogen and activators recovered in the euglobulin fraction. That is, the carrageenan in vivo may decrease the plasminogen ability to precipitate in the euglobulin fraction. Alternatively, it may increase the activity and precipitation of inhibitors, primarily $\text{C}\bar{\text{I}}$ inhibitor.

In summary, the carrageenans were observed to be fibrinolytic in vitro, with κ -carrageenan fractions having greatest activity. This activity was found to be concentration-dependent, an observation which may explain the ex vivo inhibitory action of carrageenan on fibrinolysis. The results may be related to the polymer structure of the galactan sulphates and also their electrostatic interactions with proteins and free cations.

As stated earlier, p.157, carrageenan could potentially interact with either $\text{C}\bar{\text{I}}$ inhibitor or with a plasminogen activator and $\text{C}\bar{\text{I}}$ inhibitor and plasmin could influence Factor XII-mediated responses. A direct correlation exists between Factor XII and the kallikrein-kinin system where kallikrein is known to be intimately involved in inflammatory reactions. Carrageenans have also been shown to induce inflammatory reactions and thus, the next step in the investigation centered on the kallikrein-kinin pathway.

3. Kallikrein-kinin system

The carrageenans investigated ex vivo potentiated prekallikrein-catalysed amidolysis over the initial 4 hour period but inhibited activity at 12-24 hours, the rates of amidolysis measured returning to control value at 36 hours (Graph B.3.1.1, p.120). The initial increase observed accorded with results of experiments in vitro (Graph A.3.1.1, p. 99) in which amidolysis was potentiated by the presence of alkali-modified λ - and κ -carrageenan fractions.

Kinin-releasing activity of carrageenan was also observed in vivo by Ratnoff (1966) and in vitro by Schwartz and Kellermeyer (1969) and Kindness (1979). This effect was observed to be dependent on Factor XII, prekallikrein and HMWK (van der Graaf, Koedam and Bouma, 1983). Therefore, it may be inferred that the kallikrein-carrageenan response involves a complex inter-relationship, possibly through protein macromolecular alignment in order to achieve activation of the kallikrein-kinin pathway. The provision of a negatively-charged surface with a suitable molecular architecture would form the basis for Factor XII activation. The fact that this activity is a charge effect was shown by Kindness (1979) when it was found that kinin production was inhibited when the galactan sulphate negative charges were blocked by positively charged Polybrene (hexa-dimethrine bromide).

The structure of the polymer may be important when considering charge density "gaps" along a polyelectrolyte chain. The presence of these gaps may considerably decrease the possibility and strength of protein binding (Cifonelli, 1974). The type of gel network that κ -carrageenan forms may cause masking of the negative charges, resulting

in charge density gaps; therefore, the probability of protein interactions along the chain length may be reduced compared to those possible when the less helical, or gel-like structure of the alkali-modified λ -carrageenan is involved. The ι - and κ -carrageenan structures would therefore depend on chain linking sites for protein interaction (Introduction, p. 8).

The process of kallikrein formation is strictly regulated by certain plasma proteinase inhibitors. The neutralisation of plasma kallikrein may be brought about by C $\bar{1}$ inhibitor (Ratnoff et al., 1969; Gigli et al., 1970) α_2 -macroglobulin (Harpel, 1970; Fritz et al., 1972) and antithrombin III (Burrowes, Habel and Movat, 1975; Vennerod and Laake, 1975), although AT III has little or no effect unless heparin is present (van der Graaf, Koedam and Bouma, 1983).

In summary, the amidolytic activity catalysed by prekallikrein is initially increased up to 4 hours post carrageenan dose but is inhibited after 12 hours, returning to control values at 36 hours. In vitro, only the alkali-modified λ -carrageenan and ι -carrageenan causes an increase in prekallikrein-catalysed amidolysis. These effects may be related to the ability of galactan sulphates to bind to prekallikrein inhibitors, possibly resulting in the exposure of an active site. For prekallikrein activation, there are requirements for Factor XII, HMWK, prekallikrein and a negatively charged surface, and the galactan sulphate may initiate the complex formation. This effect may, however, be blocked by antiproteinase-polymer macroion complex formation, resulting in inhibition of the kallikrein-kinin pathway.

In the study of Factor XII-mediated responses, it has been shown that many of the component parts of the coagulation cascade are found in association with the blood platelet surface (Harfenist et al., 1981).

Hence, not only has Factor XII been observed to be on the platelet surface but also other proteins such as fibrinogen. Also, the platelet contains granules capable of releasing their contents which would have a synergistic effect on thrombus formation (Introduction, p. 30). Hence, the interaction of carrageenans with platelets and possible platelet responses was investigated.

4. Platelet aggregation studies

Heparin and the carrageenan fractions had little or no effect on rat platelets in vitro. When viewed microscopically, platelets pre-incubated with high carrageenan concentrations were of normal discoid shape. Small agglutinates of platelets were seen, but no shape change or release reaction had occurred.

In view of the findings that carrageenan aggregates platelets in other species (MacMillan and Sim, 1970), the influence of the galactan sulphates in the presence of extracellular platelet aggregating compounds, namely adenosine 5'diphosphate (ADP) and thrombin, were studied. Platelets were incubated with galactan sulphates before the addition of the aggregating agent. A concentration of ADP (10 μM) which produced a reversible aggregation response was used. No significant change in the aggregation response was observed in the presence of either heparin or carrageenan and microscopic analysis showed only small agglutinates of normal discoid platelets.

ADP induced platelet aggregation in most animal species, but the degree to which this occurred varied widely (Tomikawa et al., 1978; MacMillan and Sim, 1970). In humans, baboon, cat, dog, horse and guinea pig blood, low concentrations of ADP (less than 2 μM) induced irreversible biphasic aggregation, whilst at the same, and much higher concentrations (greater than 2 μM), mouse, rat and rabbit platelets underwent reversible aggregation (Constantine, 1966; MacMillan and Sim, 1970; Nun, 1981). These results accord with those reported in this thesis.

Rat platelets, when stimulated to aggregate by thrombin (1.0 NIH U ml⁻¹), form a thrombus rapidly. At this concentration, a distinct

biphasic response was recorded (Graph A.4.3.1, p. 101). Heparin greatly reduced thrombin-induced aggregation, even at low heparin concentrations (0.001 mg ml^{-1}) (Graph A.4.3.1, p. 101). Alkali-modified λ -carrageenan showed inhibition of aggregation at 0.001 mg ml^{-1} , ι -carrageenan above 0.10 mg ml^{-1} and the K-carrageenans inhibited at 1.0 mg ml^{-1} (Graphs A.4.3.2 to A.4.3.6, p. 102 to 106).

In ex vivo assays, carrageenan fractions induced thrombocytopenia, with platelet counts decreasing to a minimum at 12 hours post dose (Graph B.4.1.1, p. 122). Carrageenan may induce rupture of the cell surface or adhere to the platelet surface to promote removal by the liver. Carrageenan at high concentrations (200 mg kg^{-1}) is known to induce disseminated intravascular coagulation, a condition which greatly reduces platelet numbers and results in severe damage to areas of blood stasis such as ears, tail and feet of the rat (Kindness - personal communication).

Alkali-modified λ -carrageenan and ι -carrageenan, when administered in vivo, inhibited platelet aggregation induced by both ADP and thrombin. ADP-induced aggregation was inhibited by the alkali-modified λ -carrageenan over a 4-36 hour period, while ι -carrageenan-treated animal plasma showed a delay before onset of inhibition at 6 hours (Graph B.4.2.2, p. 124).

Platelets have been shown to have ADP and thrombin receptors present on the cell surface, and Harfenist et al. (1981), showed that, in rabbits, platelets stimulated with ADP possessed fibrinogen binding sites. This suggests that ADP binds to platelets and induces a membrane structural change resulting in the exposure to specific and saturable receptors for fibrinogen. Prevention of fibrinogen binding to platelets by inhibitors could result in disaggregation and

dissociation of bound fibrinogen (Harfenist et al., 1981). Fibrinogen would appear to act as an adhesive, binding platelets to one another and this reinforces the concept that fibrinogen plays an important part in the initiation of ADP-induced aggregation. This phenomenon may also explain the thrombocytopenic effects observed in carrageenan injected rats.

In the presence of carrageenan ex vivo, ADP aggregation was greatly inhibited, although in vitro the carrageenans did not affect ADP-induced aggregation. A possible explanation of this effect is that the carrageenan may directly adsorb onto the platelet surface, thus preventing proteinase activity and the platelet-platelet interactions necessary for aggregation. Alternatively, since fibrinogen is a cofactor for ADP-induced aggregation, the carrageenan may act in a polyelectrolyte manner and bind to the fibrinogen molecule. Calcium is required for the final part of the coagulation cascade, and calcium ions are known to stabilise polyelectrolyte-protein complexes by neutralising free sulphate esters rather than taking an active part as a bridging cation (Lin, 1977). Also, the overall tertiary structure of the protein may allow a good "fit" into the polyelectrolyte structure as possibly occurs in the case of heparin-AT III complexes (Hopwood et al., 1976). Fibrinogen may also undergo conformational changes when the polymer is bound to the galactan sulphate fractions. This may be due to the highly charged carrageenan interacting with an oppositely charged region at the active site of fibrinogen. This would result in inhibition of fibrinogen activation and subsequent inhibition of ADP-induced aggregation.

Rat platelets, when stimulated by ADP, undergo reversible aggregation. Once the platelets disaggregate they may be further stimulated

to aggregate with ADP, illustrating that the ADP does not permanently alter the platelet surface.

Thrombin-induced platelet aggregation was inhibited by carrageenans both in vitro and ex vivo. These results may be explained by the observed results of experiments in which heparin potentiates AT III in vitro, together with previous results of in vitro experiments on AT III-depleted plasma (Graph A.1.8.1, p.87). The influence of heparin on thrombin was found to be dependent on the presence of AT III (Graph A.1.8.1, p. 87). These results suggest the formation of a heparin-AT III complex which inhibits thrombin activity and may act to protect platelets from aggregation by thrombin. It may be hypothesised that the heparin interaction with AT III present at the platelet surface greatly inhibits thrombin activation, and that a similar but weaker reaction occurs with carrageenan and AT III. These reactions may also explain why carrageenan itself does not induce platelet aggregation.

Carrageenan may interfere with platelet aggregation by a purely physical effect, in which platelet-associated calcium ions form neutralising bridges between the negatively-charged phospholipid and polyelectrolyte. The more flexible the polyelectrolyte, the more "coating" ability it has; thus the alkali-modified λ -carrageenan would be more efficient than the ι - or κ -carrageenan fractions. This was suggested in this study but could not be the only reason for ADP platelet aggregation effects since ADP aggregation in vitro was not affected by the presence of carrageenan.

The platelet adhesiveness studies ex vivo again illustrate the potential of alkali-modified λ -carrageenan and ι -carrageenan as

anti-platelet compounds. Platelet adhesiveness was found to decrease to a minimum at 4 hours after administration of either carrageenan (Graph B.4.4.1, p. 133). For each time period, the platelets were examined microscopically, and all platelets from λ -carrageenan-treated animals were observed to be normal. The alkali-modified λ -carrageenan-treated platelets ex vivo were found to have a granular appearance at 4 hours but no pseudopodia were evident, and there was no evidence of adherence or aggregation. This effect was transient, since platelets viewed at 6 hours had a normal shape and were clearly visible by light microscopy at this stage.

The mechanism of this phenomenon is unclear, but it may be postulated that carrageenan is bound to the platelet surface, and may interfere with the uptake of the dye used in the experiment. The 4 hour response may be an indication of maximal carrageenan uptake, as illustrated by the functional clotting assays. This theory assumes that carrageenan binds to platelets in a manner similar to heparin (Gogstad, Solum and Knitnes, 1983).

The delay in onset of inhibition by λ -carrageenan was observed in most assays carried out. The alkali-modified λ -carrageenan inhibited coagulation and had an anti-platelet effect earlier in the time course than λ -carrageenan. Alkali-modified λ -carrageenan may be taken up more readily by the peritoneal blood circulation than the more gel-like λ -carrageenan. Alternatively, the lower charge density of λ -carrageenan may reduce the possibility of protein alignment. Although each polyelectrolyte has a high sulphate content, the availability and orientation of these groups is important. Also, as with the heparin-AT III complex already discussed (p. 153), a set configuration of disaccharide repeat units may be required for protein-carrageenan

interaction.

In summary, carrageenans in vitro induced no aggregation, as well as having no effect on ADP-induced aggregation. Thrombin-induced aggregation was inhibited both in vitro and ex vivo by carrageenans, an effect which may occur via direct physical binding of carrageenan to the platelet surface or via interaction with platelet membrane-bound AT III to form an active complex. ADP-induced aggregation ex vivo was inhibited by carrageenan, possibly via fibrinogen-carrageenan complex formation.

Delay of onset of the inhibitory effect on aggregation may be a reflection of the physical nature of the carrageenans, with the alkali-modified λ -carrageenan having an effect earlier in the time course than ι -carrageenan. Both carrageenans ex vivo caused platelet thrombocytopenia and inhibited platelet adhesiveness, possibly due to carrageenan-platelet complex formation via calcium bridges.

It may be possible for a carrageenan-immunoglobulin interaction to take place where the immunoglobulin acts as an aggregating agent. Thus, the next stage in the investigation was to examine carrageenan-immunoglobulin interactions.

5. The immune system

Carrageenans have been used frequently as tools to study the immune response and the reticuloendothelial system. Few investigators, however, have tried to relate the immune response to carrageenan-induced Factor XII-mediated haemostatic responses. The nature and magnitude of the effects of carrageenans were found to be dependent on both the route of injection and the form and purity of the material. In this study, the i.p. route of administration was used to allow correlation with the coagulation studies and assay results showed this route to give a stronger immunosuppressive effect over other routes (Cruickshank, personal communication). The characterised carrageenans were used to investigate their effects on the immune system but alkali-modified λ -carrageenan was observed to have little immunosuppressive effect in the rat. Immunosuppression was found with all concentrations of λ -carrageenan but maximally at 40 mg kg^{-1} when injected 24 hours prior to antigen, as shown by the dose response curve (Graph B.5.2.1, p. 137). The time of carrageenan administration in relation to antigen suggested that the carrageenan must exert an effect on the afferent limb of the immune system and not on the antibody producing B lymphocytes. The carrageenan may act at an early stage in the processing of the antigen by macrophages or in the macrophage-lymphocyte interaction (Lake, Bice and Schwartz, 1971).

A repeat of this study revealed considerable variability in responses, both to antigen alone and to the effects of carrageenan on the immune response. The reasons behind these variable responses remain unknown.

Rats injected with $40\text{--}60 \text{ mg kg}^{-1}$ λ -carrageenan i.p. developed certain pathological conditions. In all animals the caudate lobe of

the liver was enveloped in a loosely bound capsule, and small plaques of the same material were loosely adherent to the liver surface. Microscopic examination of these plaques showed that a high percentage of the cell population consisted of actively proliferating fibroblasts containing carrageenan. Large macrophages containing carrageenan were seen by staining the preparation with corphosphine so that the presence of acid mucopolysaccharides on the cell surfaces was indicated as a light orange colour; large areas of bright orange were considered to represent the presence of carrageenan. The sac surrounding the caudate lobe of the liver consisted of a mesoendothelial sheath containing a proliferating mass of connective tissue. Many macrophages contained carrageenan globules coalescing around the nucleus, while other macrophages had actively undergone cell fusion. Cellular debris suggested that some macrophage destruction had occurred.

Immediately below the mesoendothelial sheath, dense masses of carrageenan were present around which macrophages were seen to aggregate.

The inflammation within the duodenum was also studied histologically. The circular musculature was seen to be intact but the inflammatory response had induced a proliferation of connective tissue similar to that of an epitheloid granuloma. This region contained microvasculature into which many polymorphonuclear leucocytes had migrated. As in the liver, there was evidence of cell fusion and carrageenan uptake by both macrophages and fibroblasts.

No such pathological changes, but substantial immunosuppression, was evident at 20 mg kg^{-1} and for all further studies this concentration of 1-carrageen was used.

Finally, to evaluate the effect of time of carrageenan administration in relation to antigen, 1-carrageenan was injected i.p. pre- and post-SRBC. Variability in the response was observed, and by using regression analysis no relationship was determined. These results remain somewhat of an enigma, since in all cases the animals were of the same sex, breeding stock and were maintained under the same housing conditions and diet; all these factors can affect immunoglobulin levels.

Carrageenan has been previously described as an irritant which is toxic to macrophage, resulting in eventual cell death in vitro (Allison, Harrington and Birbeck, 1966; Cateinzaro, Schwartz and Graham, 1971). Recently, however, administration of carrageenan to the lung illustrated that an increase in lung macrophage longevity occurred (Bowers, Stapleton and Lew, 1983). These authors observed that carrageenan induced an inflammatory response which was characterised in its early stages by immediate polymorphonuclear leucocyte infiltration. After 2 days the polymorphs were replaced by macrophages bearing carrageenan. Macrophages, which readily take up carrageenan, swell due to the inability of the cells to degrade the carrageenan (Pugh-Humphreys, personal communication). Rupture of some macrophages in the peritoneum was observed in the present study, with the migration of cellular material to the liver region resulting in the formation of adherent plaques. Within these plaques large macrophages containing carrageenan or other polysaccharide were evident. These macrophages may have remained viable or may be a secondary phase sequestering cellular debris and carrageenan. The incorporation of carrageenan into macrophages without cell death may result in increased macrophage longevity as initially hypothesised by Spector

(1974) and may be the mechanism of maintained inflammation. These macrophages may be prevented from leaving the peritoneum by purely physical characteristics because carrageenan uptake into the macrophage lysosomes causes an increase in cell size.

Kindness (personal communication) showed that, at high carrageenan concentrations, rat tail, ear and paw damage occurred primarily in κ -carrageenan-treated animals. Such damage was also observed in rat by Thomson et al. (1976) using potassium carrageenan (50 mg per animal). Clearly, however, the underlying cause of such damage is disseminated intravascular coagulation (DIC) caused either by a direct effect of carrageenan on platelets, inducing thrombocytopenia (Graph B.4.1.1, p. 122), or by an effect of carrageenans on rat red cell membranes (Pittz, Golberg and Coulston, 1975).

Girman et al. (1976) found that FDPs, when administered at the same time as, or 24 hours after, antigen, induced immunosuppression in vivo. However, in the present study, ι -carrageenan inhibited fibrinolysis (Graph B.2.1.1, p. 115) and therefore no relationship between FDP induction and the observed immunosuppression can be suggested.

When macrophages encounter inflammatory stimuli in vivo they respond by releasing a number of products which may account for the central role that these cells have in chronic inflammatory diseases (Davies and Allison, 1976).

Active phagocytosis induces the production of a lymphokine called Interlukin 1. This interacts with the circulating T cells which in turn are stimulated to produce Interlukin 2 which acts to suppress B cell immunoglobulin synthesis. Since SRBC are T cell-dependent

antigens this may have a direct relationship to the suppressive effects observed in this study (Watson, Mochizuki and Gillis, 1980; Oppenheim and Gery, 1982; Altman, 1982).

6. Lipoprotein studies

Lipoproteins were separated from plasma obtained from carrageenan-treated rats by density ultracentrifugation. The concentration of lipoprotein was estimated using a cholesterol assay and results illustrated on Graphs B.6.1.1 to B.6.1.4 (p. 143 to p. 166) show that alkali-modified λ -carrageenan (20 mg kg^{-1}) caused an increase in VLDL and LDL concentrations at 12 hours post dose while not affecting IDL and HDL. With γ -carrageenan, LDL concentrations were increased but VLDL, IDL and HDL concentrations were not significantly affected.

Of the lipoproteins, VLDL contain least cholesterol and as the density of the lipoprotein increases cholesterol content also increases. This phenomenon was found in this study when control values over the time course were found to be $6.1 \pm 0.7 \text{ mg } 100 \text{ ml}^{-1}$ serum in VLDL to $30.4 \pm 1.8 \text{ mg } 100 \text{ ml}^{-1}$ with HDL (Graphs B.6.1.1 and B.6.1.4, p. 143 and p. 146).

The alkali-modified λ -carrageenan appears initially to cause the release of lipoprotein lipase from surface-bound receptors in peripheral tissue in a heparin-like manner. Heparin, when administered i.v., showed antilipemic activity 10 minutes after injection, an effect which has been shown to last from 30 minutes to 2 hours (Mahadoo, Wright and Jaques, 1981; Bianchini et al., 1982). The action of heparin appears to be mediated through the release of lipoprotein lipase from endothelial receptors, thus changing the site of lipoprotein hydrolysis from limited segments of the capillary bed to circulating blood (Engelberg, 1978). Heparin then prevents lipoprotein lipase binding to the receptors on the cell surface by occupying the receptor site (Goldstein et al., 1976).

The unusually high concentrations of VLDL and IDL observed with alkali-modified λ -carrageenan at 0 and 2 hours were unexpected. Comparison with control values suggest that the animals used had high levels of VLDL and IDL similar to these observed prior to feeding of a high cholesterol diet. For every experiment the animals were starved for no less than 24 hours prior to carrageenan injection, and administration of polysaccharide was always carried out in the early morning. The significant decrease to control values at 4 hours suggest lipoprotein lipase activity, but concomitant LDL or HDL increases were not observed (Graphs B.6.1.3 and B.6.1.4, p. 146 and p. 147).

Rats are unique in that they have low LDL levels due to rapid clearance of this lipoprotein by the liver (Lasser et al., 1973). The liver plays an important part in rat sterol synthesis, while in other species studied (rabbit, guinea pig, hamster and monkey) the extrahepatic tissues are quantitatively more important (Koelz et al., 1982). The LDL turnover is relatively rapid with a reported half life of 4.3-8.5 hours (Hay et al., 1971) while plasma cholesterol is carried by the HDLs (Lasser et al., 1973). It should be noted, however, that LDL harvested from rat serum by conventional ultracentrifugation techniques may be contaminated with HDL, and quantitative errors may result in the measurement of LDL concentrations. Analysis of contamination could be carried out by separation of the apoproteins to find levels of HDL apoprotein E.

The λ -carrageenan results show that little effect was observed on VLDL concentrations, although a small but significant peak was observed with IDL at 6 hours (Graph B.6.1.1 and B.6.1.2, p. 144 and p. 145). An increase in IDL may occur because lipoprotein lipase activity on

VLDL causes a transient increase on IDL levels. The effect seen at 6 hours would correspond to 1-carrageenan uptake, as found by the functional clotting assays. The rise in IDL, although significant, was sufficiently small that no direct decrease in VLDL levels may be recorded. However, the LDL level was increased at 6 hours, suggesting that accumulation of these lipoproteins may be occurring (Graph B.6.1.3, p. 146). This hypothesis may be countered by the fact that the half-life of LDL is 4-8 hours while that of HDL is 8-11 hours (Lasser et al., 1973). Hence some other mechanism must be influencing these lipoproteins causing an increase in their concentration.

It may be that the carrageenan promotes synthesis of LDL by the liver, a situation found to occur in rooster by Behr et al. (1981). Similarly, sulphated polysaccharides could bind lipoproteins by ionic interactions (Laurent, Waserson and Öbrink, 1969). This may cause a retention of LDL by the circulation since receptor sites on the liver may no longer recognise the LDL-polyelectrolyte complex as containing LDL. Thus, 1-carrageenan may bind IDL and LDL at the point of maximum carrageenan uptake due to its gel network structure and ionic binding properties. Rapid removal of the carrageenan would occur within the 24 hour period, as reflected by the functional assays, thus returning lipoprotein concentrations to normal. The possibility that sulphated GAGS bind LDL was also suggested by Donnelly, Di Ferrante and Jackson (1978).

It has also been shown that the presence of glycosaminoglycans promotes physical changes in LDL (Bihari-Varga, Sztatisz and Gál, 1981). At body temperature, LDL is in solution, but on addition of glycosaminoglycan a complex forms between the glycosaminoglycan and LDL, rendering the LDL crystalline. This may possibly result in decreased

mobility, increased resistance to enzymes and possible deposition on artery walls. If, however, a significant amount of HDL is present the GAG-LDL complex remains in solution. The mechanism of this phenomenon is not fully understood (Bihari-Varga, Sztatisz and Gál, 1981).

These results suggest that instead of polysaccharides having an antilipemic effect they may be an additional risk factor for atherosclerosis. This may be true for humans, but the induction of an atherosclerotic state in rats may be difficult, due to the fact that the above interactions rely on LDL binding. As previously stated, LDL in rats is rapidly cleared and cholesterol is transported by HDL, which probably has less affinity for polysaccharide due to a potential lower ionic interaction. Hence the induction of an atherosclerotic state may be prevented by this unique LDL removal system (Lasser et al., 1973).

The effect of the alkali-modified λ -carrageenan occurs principally on the VLDL and IDLs, with a rapid reduction in VLDL s at 4 hours but a rise at 12 hours (Graph B.6.1.1, p. 144). The alkali-modified λ -carrageenan may act in a similar manner to heparin by releasing lipoprotein lipase and by binding to hepatocytes to facilitate the uptake of the remnants produced by lipase activity (Goldstein et al., 1976).

As the carrageenans may act in a heparin-like manner to cause lipoprotein lipase release from the endothelium, it may be presumed that lipase activity would become exhausted, resulting in an increase in VLDL concentration (Graph B.6.1.1, p. 144).

In summary, the initial high concentrations of VLDL and IDL found in the alkali-modified λ -carrageenan-treated animals require

further study. The alkali-modified λ -carrageenan was shown to have an antilipemic effect, possibly due to the release of lipoprotein lipase from the endothelial cells induced by carrageenan competitive binding. An increase in VLDL concentrations at 12 hours may reflect a lipase exhaustion and retention of the VLDL concentration in the circulation. Little or no effect was observed on the other lipoprotein concentrations.

λ -Carrageenan was found to affect primarily the LDL and HDL concentrations and it may be postulated that this occurs by an ionic interaction between the carrageenan and the lipoproteins, possibly causing a change in the physical behaviour of the lipoprotein preventing removal by the liver.

VI CONCLUSION

The proteinase systems of blood which mediate inflammation and haemostasis, that is, the kallikrein-kinin, fibrinolytic and coagulation pathways together with the blood platelets, are usually considered in isolation, perhaps because of the differing techniques initially applied to their study and because it was conceptually easier to consider them separately. With increasing recognition of the complexity of the systems has come an appreciation of their inter-relationships, although the functions of the various points of interconnection are still being studied.

The pathways studied within this thesis are principally activated by one proteinase, Factor XIIa, which in concert with HMWK and pre-kallikrein forms the focal point of this study. As Figure 2.8.1, p. 35 illustrates, the pathways are regulated by positive and negative feedback mechanisms arising from, and inter-relating with, other pathways. This results in a steady state in which vessel potency and blood fluidity is maintained.

Carrageenans are anionic polyelectrolytes, which in physiological conditions of pH 7.4 and ionic strength of 0.15 mS cm^{-1} , may bind some of the many plasma proteins that have a net positive charge under these conditions. Several factors are important in this binding, such as the three-dimensional shape and gel-forming abilities of the galactan sulphates together with their electrostatic and hydrogen bonding properties. Alignment of the protein may not be total, and intermediate degrees of association may occur. Ions present within the plasma may be not only required for the binding between positive and negative charged components but may also stabilise complexes which

form by binding to unbound charged groups on either molecule. Alternatively, these cations may act as bridges between two negatively-charged units such as carrageenan and platelet phospholipid or the complexes required for coagulation factor activation.

In summary, the important features for charge interaction are the concentration and mixing ratios of the polyanion and polycation, the pH, the concentration and charge of electrolytes, the charge density, nature, spacing and stereochemistry of the charged groups, and the molecular weights of the polymers.

Alkali-modified λ -carrageenan and ι -carrageenan both inhibited in vitro and ex vivo coagulation, through inhibitory effects on Factor XIIa and thrombin. These effects may be exerted partially via interactions with antiproteinase such as AT III. In vitro κ -carrageenan had little or no effect on coagulation but had potent fibrinolytic effects, results not found ex vivo with any fraction. In contrast, the carrageenans studied ex vivo inhibited fibrinolysis.

Carrageenans alone did not affect platelets, nor did they affect ADP-induced aggregation in vitro but administration of carrageenan to rats inhibited ADP-induced aggregation. Aggregation effects due to thrombin were inhibited both in vitro and ex vivo, an effect which was maximal at 4-12 hours post dose. Carrageenans ex vivo also induced thrombocytopenia and reduced platelet adhesiveness. The ex vivo response to carrageenan was found to be time dependent. The onset of the response may reflect the chemical nature of the polymers with the alkali-modified λ -carrageenan exerting its effect more rapidly than the ι -carrageenan. Again this may be an inherent property of the absorption and distribution of these polymers from the peritoneum.

Of the two fractions studied ex vivo, alkali-modified λ -carrageenan affected lipoprotein levels probably through the displacement of lipoprotein lipase from the endothelial cell surfaces. In contrast, while ι -carrageenan had little effect on lipoprotein concentrations, this fraction was found to be immunosuppressive while the alkali-modified λ -carrageenan had no effect.

The interaction processes involved in these pathways is complex but results obtained within this thesis illustrate that carrageenans may act to modify the following systems: the intrinsic pathway of coagulation; kallikrein-kinin pathway; fibrinolytic pathway; platelet responses; and immunoglobulin synthesis in response to antigen. Factor XII activation by carrageenan occurs in conjunction with the fibrinolytic and kallikrein systems but in the coagulation system this effect is masked by the effect of carrageenan at other areas of the coagulation pathway. Several proteinase inhibitors are common to the pathways investigated and the carrageenans could influence these proteins to modify their activity. In the case of AT III, a direct interaction with carrageenan may be postulated although this did not account for the total anticoagulant effect obtained in the thesis.

Further studies are required to pinpoint the exact areas at which carrageenan exerts an effect on the systems studied within this thesis.

In conclusion, by careful selection of concentration, structure, mode of injection and time course, these galactan sulphates may serve as useful exploratory tools in the elucidation of polyelectrolyte-protein interactions.

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